

A STUDY OF SOME METHODS INVOLVING
THE DETERMINATION OF HYDROGEN PEROXIDE ,
IN PARTICULAR IN FLOW INJECTION ANALYSIS



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A STUDY OF SOME METHODS INVOLVING THE DETERMINATION OF HYDROGEN PEROXIDE, IN PARTICULAR IN FLOW INJECTION

ANALYSIS

by

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Abstract Analytical methods involving the <u>determination of hydrogen peroxide</u> were developed for application to industrial, clinical and biotechnological samples. Hydrogen peroxide was determined by <u>electrochemical oxidation</u> of the peroxide at <u>glassy carbon electrodes</u> either by <u>linear sweep voltammetry</u> or by <u>amperometric detection in flow injection analysis (FIA)</u> systems. The FIA methods were intended for continuous monitoring of the hydrogen peroxide in <u>pickling baths</u> for copper and copper alloys and in a biotechnological process. Automated FIA-systems containing <u>immobilized-enzyme reactors</u> , columns for sample treatment and <u>on-line dialysers</u> were developed for the determination of <u>D-galactose</u> in <u>serum</u> and <u>urine</u> as well as the determination of certain other <u>carbohydrates</u> acting as substrates for the enzyme <u>galactose oxidase</u> . The hydrogen peroxide produced in the enzyme catalyzed reaction was determined either <u>spectrophotometrically</u> or <u>amperometrically</u> .			
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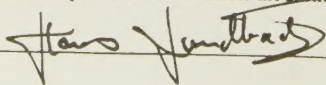
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A STUDY OF SOME METHODS INVOLVING THE DETERMINATION OF
HYDROGEN PEROXIDE, IN PARTICULAR IN FLOW INJECTION
ANALYSIS

This thesis is based on the following papers, referred to in the text
by their Roman numerals.

- I Determination of Hydrogen Peroxide in Pickling Baths for Copper Alloys by Linear Sweep Voltammetry.
Hans Lundbäck and Gillis Johansson
Anal. Chim. Acta, 128 (1981) 141
- II Amperometric Determination of Hydrogen Peroxide in Pickling Baths for Copper and Copper Alloys by Flow Injection Analysis.
Hans Lundbäck
Anal. Chim. Acta, 145 (1983) 189
- III Determination of Hydrogen Peroxide for Application in Aerobic Cell Systems Oxygenated via Hydrogen Peroxide.
Hans Lundbäck, Gillis Johansson and Olle Holst
Anal. Chim. Acta, 155 (1983) 47
- IV Galactose Determination in an Automated Flow Injection System Containing Enzyme Reactors and an on-line Dialyser.
Bo Olsson, Hans Lundbäck and Gillis Johansson
Submitted to Anal. Chim. Acta.
- V Amperometric Determination of Galactose, Lactose and Dihydroxyacetone using Galactose Oxidase in a Flow Injection System with Immobilized Enzyme Reactors and on-line Dialysis.
Hans Lundbäck and Bo Olsson

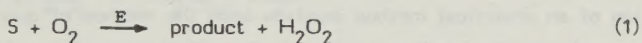
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1. INTRODUCTION

Hydrogen peroxide has for a long time been known for its decolourizing properties and found a wide use in food stuff industry and cosmetics. Large quantities of hydrogen peroxide for bleaching purposes are also consumed in the cellulose and textile industries. The oxidizing properties of hydrogen peroxide have also been utilized in organic synthesis and in polymer production. Its use in steel and nonferrous alloy factories is increasing partly because hydrogen peroxide is replacing chromates for environmental reasons. The uses of hydrogen peroxide as a sterilant for food packing materials and as bactericide in the sugar industry have been increasing rapidly in the past few years. The possibilities of utilizing hydrogen peroxide as an oxygen source in biotechnological processes have also been discussed.

The use of enzymes makes it possible to develop very sensitive and selective analytical methods for compounds which otherwise are not readily detected with common methods. Many enzymes (E), e.g. oxidases, catalyze a reaction where a substrate molecule (S) is aerobically oxidized with the cocomittant production of hydrogen peroxide.



At least forty oxidases that produce hydrogen peroxide are known [1,2]. Several of these have been used for analytical applications e.g. in clinical methods for cholesterol, uric acid and particularly glucose. Multi-enzyme systems including hydrolysing enzymes may make detecting devices for hydrogen peroxide attractive also for polysaccarides in the starch and cellulose industry.

A very large number of methods for detecting moderate and high concentrations as well as minute quantities of hydrogen peroxide is described in the literature, see Table I. No single method is generally applicable for all purposes. Rapid routine methods for handling large quantities of samples are required in clinical chemistry and for automatic monitoring and control of industrial processes. The research in this work has therefore been directed towards detecting systems which will be easy to adapt for automation.

Table I. Common methods for detection of hydrogen peroxide.

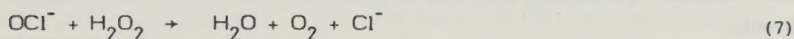
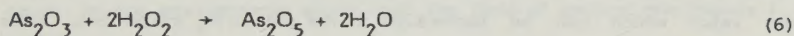
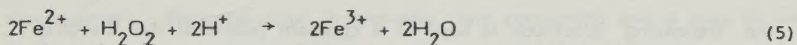
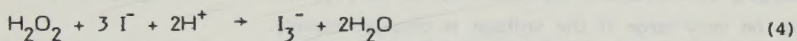
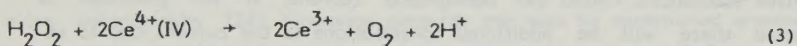
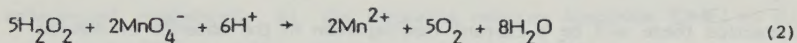
		Ref.
Titrimetry		(3-9)
Direct electrochemistry	Amperometry	(10-21)
	Voltammetry	(22-26)
	Coulometry	(27)
By chemically coupled reactions giving species measured by:	Spectrophotometry	(28-43)
	Amperometry	(44-51)
	Coulometry	(52-54)
	Potentiometry	(55-57)
	Fluorometry	(58-63)
	Chemiluminescence	(64-73)
By disproportionation of hydrogen peroxide:	Oxygen measurements	(74-76)
	Thermometry	(77,78)

Automation of an analytical method involves both the method of quantitation and the sample handling and pretreatment. Quantitation can be performed by a number of instrumental methods working with optical, electrochemical or thermal detection. The sample handling can in theory be mechanical but due to the complexity of the construction it has rarely been used except in very large volume analyses e.g. in clinical laboratories. Flow methods offer a very simple approach and air-segmented analyzers have found wide spread uses in medicine and industry. Injection into a nonsegmented carrier has gained wide spread acceptance in recent years. It offers a number of advantages in particular in flow manifolds containing packed-bed reactors.

2. DETERMINATION OF HYDROGEN PEROXIDE

2.1 Titrimetry

Quantitative determinations of hydrogen peroxide by volumetric methods are well established techniques that can give very precise and accurate results. A number of titrants is available, the most commonly used reactions are [2-8]



The completion of the reaction can be determined visually or potentiometrically or excess reagent can be added and back-titrated.

2.2 Direct electrochemical detection

Hydrogen peroxide is an electroactive species, which can be either oxidized or reduced at an electrode. Amperometric methods for the determination of hydrogen peroxide have been extensively used and they will be discussed in the following.

2.2.1 Amperometry

"Basic principles"

Amperometry is a generic term for techniques whereby the concentration of an electroactive substance is measured by the current which results from its reaction at an electrode. Ideally, the current, i , will thus be a

function of fundamental constants and the concentration gradient $(\frac{\partial C}{\partial x})_{x=0}$ of the substance at the surface, i.e.

$$i = nFAD \left(\frac{\partial C}{\partial x} \right)_{x=0} \quad (8)$$

n is the number of electrons in the overall electrode reaction, F is Faraday's constant, A is the electrode area, x the distance from the electrode and D the diffusion coefficient of the reactant [79].

In practice there will be a current flowing even in the absence of electroactive substances, called the background current. If the potential is varied there will be additional contributions to the current due to recharging of the double layer and the surface groups. The charging current may be very large if the voltage is changed rapidly.

If the indicating electrode is kept at a constant potential the charging current will quickly decay and the background current will reach a steady-state value which can be backed-off in the measuring instrument. Under these conditions the current can thus be a direct measure of the Faradic current.

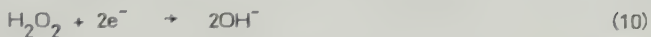
The concentration gradient $(\frac{\partial C}{\partial x})_{x=0}$ in eq(8), depends on the mode of mass transport in the solution and on the applied voltage. If the electrode is made sufficiently oxidizing or reducing all electroactive material reaching the surface will react and the concentration will then become zero at $x = 0$. If the mass transport has reached its steady-state value, eq(8) can then be written

$$i = n F A D K C^* \quad (9)$$

where K is a constant depending on many variables, e.g. the mode and rate of mass transport, and C^* is the bulk concentration of the electroactive substance.

2.2.2 Reduction

Hydrogen peroxide can electrochemically be reduced at all common electrode materials. The reaction which formally can be written



is highly irreversible. This reaction has been studied extensively at both metal [80,81] and carbon electrodes [82,83]. Hydrogen peroxide can be determined analytically at the dropping mercury electrode (DME) by d.c. polarography [22], by differential pulse polarography [23], or by reverse pulse polarography [24]. Hydrogen peroxide can also be determined amperometrically at a platinum electrode held at a constant potential [10].

In all these methods oxygen present in the sample has to be removed before measurement. Deoxygenation is normally time consuming and it may be inconvenient if the method is intended for flow analysis [84]. Metal ions will cause severe interferences which limit the field of applications (papers I and II).

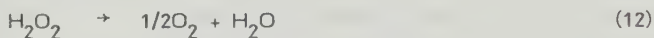
2.2.3 Oxidation

The mechanism of electrochemical oxidation of hydrogen peroxide in aqueous solutions has been a subject of continuous studies for over a century. The overall reaction, which in acid medium can be written as



gives two electrons and one molecule of oxygen [85]. Although it is quite irreversible, especially in neutral and acid solutions, the reaction will proceed quantitatively according to equation (11) at most common electrode materials [86] provided that the non-electrochemical decomposition can be neglected. The mechanism as well as the kinetics of the oxidation are complex and dependent on the reaction conditions, i.e. the type of electrode material, the pH, the buffer constituents and the concentration of hydrogen peroxide. The activity state of the electrode is important and the current will therefore depend on the prehistory and the treatment the electrode has been subjected to [87].

Platinum electrodes show a specific behaviour, which is attributed to chemical interaction between hydrogen peroxide and platinous oxides on the surface [80,86]. These oxides (e.g. PtO), which begin to form around +0.5 V vs SCC at neutral pH, are easily reduced by the hydrogen peroxide. This is probably why the overpotential for the oxidation of hydrogen peroxide is less at platinum than at gold or carbon electrodes. The platinum electrode itself will be oxidized to finely divided platinum which accumulates with time. It will increase the rate of the catalytic decomposition of hydrogen peroxide



The rate of eq(12) has been shown to be nearly as fast as the electrochemical oxidation, eq(11) [27]. Effects like those mentioned may lie behind the poor reproducibility observed with the amperometric [11] or voltammetric (paper I) methods for determination of hydrogen peroxide at high concentrations. Detection devices based on direct electrochemical oxidation of hydrogen peroxide at platinum electrodes have been used in combination with immobilized enzymes [12-19,88]. The catalytic decomposition is probably of little importance at these low concentrations. Micro-electrodes with immobilized glucose oxidase have been discussed as implantable glucose sensing devices [20].

Side reactions are also possible at carbon electrodes, e.g. formation of carbonyl groups [89] or carboxyl groups [90]. The formation of such groups becomes important at the high positive potentials needed for the oxidation of hydrogen peroxide at e.g. glassy carbon electrodes (papers I, II, III). The surface state of the electrode will change and the current will thus change with time.

In spite of the irreversibility of the reaction and the presence of time-dependent side reactions which are only partially understood it is possible to select the conditions for the direct oxidation of hydrogen peroxide so that an accurate, sensitive and reproducible determination can be made. Empirical electrode pretreatment schemes are necessary in order to succeed with such an approach (papers I, II, III). It was found that interferences from metal ions and certain organic compounds were negligible at the glassy carbon electrode in contrast to the interferences at noble metal elec-

trodes. A commercial instrument developed for glucose analysis is based on amperometric oxidation of hydrogen peroxide at a glassy carbon electrode [21].

2.3 Indirect detection

2.3.1 Introduction

Indirect methods for detecting hydrogen peroxide are mostly based on the reaction between the peroxide and a compound the oxidized form which is more readily detected than the peroxide itself.



The final method of detection is then of course selected with respect to the used substrate (Table I). A catalyst is commonly used in order to make reaction (13) fast and quantitative. A large number of inorganic, organic and biochemical catalysts has been used. The choice of catalyst depends on the kind of substrate and on the detecting technique. Some catalysts are listed in Table II together with the applied methods of detection.

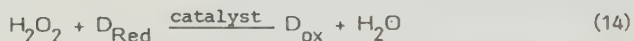
Table II. Some catalysts used in the detection of hydrogen peroxide by spectrophotometry (S), electrochemistry (E), fluorometry (F) or chemiluminescence (CL).

Catalysts		Detection method	Ref
Metal ions:	Mo(VI)	S,E	(28,55)
	Cu(II)	S,CL	(29,68)
Metal ion complexes:	Fe(III)CN ₆	CL	(69)
	Cu(II)-histamine	S	(30)
	Co(III)-tri-ethanolamine	CL	(70)
Heme containing compounds:	Peroxidase	S,E,F,CL	(31,56,60,71)
	Microperoxidase	CL	(73)
	Hematin	CL	(72)
	Catalase	S	(32)

The enzyme peroxidase (POD) is very selective towards hydrogen peroxide and it catalyzes the oxidation of a great variety of both inorganic and organic substrates. The versatility of peroxidase has made it useful as a catalyst in combination with many detection methods. Peroxide was used in this work with amperometric (paper V) or spectrophotometric (papers III and IV) detection.

2.3.2 Spectrophotometry

The basic reaction for spectrophotometrical detection of hydrogen peroxide is



where D_{Red} and D_{ox} are the reduced and the oxidized forms of the chromogen respectively. The absorbance obtained from the coloured product D_{ox} will thus be a measure of the concentration of hydrogen peroxide.

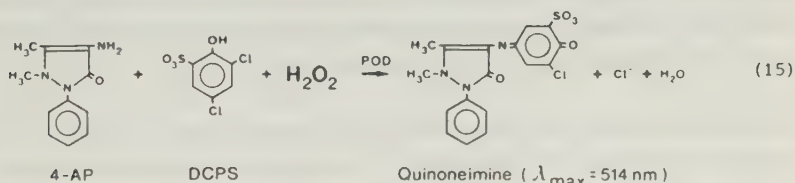
A large number of compounds has been investigated as chromogens in reaction (14) and the choice of a suitable chromogen should be based on the requirements for each special case. The selectivity of the method is dependent on factors such as the wavelength maximum and the molar absorptivity of the coloured product D_{ox} as well as resistance towards chemical interferences from other compounds present in the sample matrix. Chemical interferences and their removal will be discussed in section 3.3.

A good colour reagent has to be non toxic, stable and be a good substrate for e.g. peroxidase. The coloured product (D_{ox}) should have a favourable wavelength maximum (λ_{max}), a high molar absorptivity, be stable and show resistance towards reduction. A good substrate should also give a quantitative yield of coloured product and the reaction should proceed at a sufficiently high rate. Short reaction time is required for rapid analysis and this is especially important in flow injection analysis (FIA).

Many of the classical inorganic chromogens such as titanium(IV)sulfate [33], iodide [34], hexacyanoferrate(II) [35] etc. suffer from poor sensitivity or inconveniently low λ_{max} of the coloured product and are thus less suitable for e.g. biological samples. These compounds have therefore

been replaced by other chromogens of which aromatic amines or aromatic phenols are the most frequently used. The oxidation products are highly coloured and they absorb at wavelengths less susceptible to spectral interferences. These chromogens can be divided into two main classes representing two different reaction types. The first class includes certain types of redox indicators, such as o-dianisidin [36] and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [37] which upon oxidation give a coloured dimer or a stable radical respectively. The second class is characterized by oxidative coupling of an aromatic amino compound with another aryl compound. The latter class includes the oxidative coupling of 4-aminoantipyrine (4-AP) or 3-methyl-2-benzothiazolinone hydrazone (MBTH) with either an aromatic phenol or an aromatic amine [91-93].

A reagent described by Trinder [31] but later modified [39] and widely used by many workers [39-43], was employed in paper III for the determination of hydrogen peroxide in solutions containing high concentrations of dihydroxyacetone and glycerol. The reagent consists of 4-AP and a substituted phenol 2,4-dichlorophenol-6-sulfonate (DCPS) and the analytical reaction is outlined below (eq(15)).



The same reagent was used for the development of a method for the determination of galactose in serum by FIA.

2.3.3 Indirect electrochemistry

In indirect electrochemistry the compound D_{Red} in eq(7) is converted to D_{Ox} and the couple $D_{\text{Red}}/D_{\text{Ox}}$ constitute an electrochemically reversible redox pair making the choice open which electrochemical detection system to be used.

The most widely used mediator (D_{Red}) is iodide but several other mediators including hexacyanoferrate(II), bromide etc. as well as quinoic or phenolic organics have been used.

Iodide reacts with hydrogen peroxide with the liberation of iodine and the change in redox potential of the solution can be indicated by a platinum electrode [55]. The change in redox potential is directly correlated with the amount of peroxide in the sample. As with all redox potential measurements, other redox couples will cause severe interferences. Ion selective electrodes and especially the iodide selective electrode are much less sensitive to this type of interference. Iodide selective electrodes have conveniently been employed in continuous flow analysis for determination of glucose [56] and cholesterol [57] via the enzyme catalyzed production of hydrogen peroxide. One drawback with the ion selective electrode approach is that the decrease in iodide is measured and the dynamic range of the method will therefore be small. The detection limit will be of the order of 10^{-4} M H_2O_2 .

Methods grounded on current or electric charge measurements are considerably more sensitive than potentiometric ones and many such methods have been developed for the detection of hydrogen peroxide. They are based either on coulometric or amperometric principles.

Coulometric methods are recognized by good accuracy and precision. Coulometric reagents including iodide [52,53] and bromide [54] have been used in methods for hydrogen peroxide based on reaction (14). (Methods based on the chemical oxidation of hydrogen peroxide by a strong oxidant exist as well [94,95]).

Most coulometric methods suffer from complexity and analysis times of several minutes are required. They are not easily adapted to flow analysis. However, new efficient flow through coulometric detectors have been developed and some have appeared on the market quite recently. These may make coulometric methods for hydrogen peroxide attractive again for flow analysis.

The amount of iodine formed in the oxidation of iodide by hydrogen peroxide can be measured amperometrically [44-46]. Hexacyanoferrate(II) [47-49] and

certain organic compounds [50] have also successfully been employed as reagents. Amperometry is inherently a very sensitive technique, particularly when applied to flow analysis and this has been demonstrated by several applications [47,50,51]. In paper V hexacyanoferrate(II) and an organic mediator, methylcatechol, were used for amperometric detection of hydrogen peroxide. The FIA-system was developed for the enzymatic determination of some carbohydrates of clinical and industrial interest. Iodide was excluded as reagent since iodine was found to interact with the immobilized peroxidase enzyme.

2.3.4 Fluorometry

Fluorometric analysis of hydrogen peroxide depends on the peroxidase catalyzed production of a fluorogenic compound in a reaction analogous to eq(14). Upon excitation with light of the appropriate wavelength the compound D_{ox} becomes fluorescent and emits light at a longer wavelength. The energy of the emitted light is directly proportional to the concentration of hydrogen peroxide.

A wide variety of hydrogen-donating substrates produces fluorogenic substances when coupled to the peroxidase catalyzed oxidation of hydrogen peroxide. Many of these compounds have been employed for the assay of hydrogen peroxide and for coupled assays of compounds of clinical importance such as glucose, cholesterol, uric acid etc. [96]. In the search for new highly fluorogenic substrates 4-hydroxyphenyl compounds have been discovered to be very sensitive [59]. Detection limits in the order of 10^{-8} M H_2O_2 are obtained. Fluorometric methods are seriously affected by quenching from highly absorbing species or interfered by compounds that fluoresce at the detection wavelength. These methods are also affected by the same chemical interferences present in the spectrophotometric methods (see section 3.3).

2.3.5 Chemiluminescence

Chemiluminescence arises when hydrogen peroxide reacts with a certain reagent to produce an electronically excited state which emits light when returning to the ground state. This technique has proved to be very sensitive with detection limits in the order of 10^{-9} - 10^{-8} M H_2O_2 .

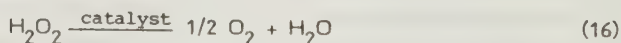
Depending on the type of reagent, the chemical reaction has to be catalyzed by a suitable catalyst, sensitized by a fluorescer or both [97,98].

The luminol reaction has been the most widely used for detection of hydrogen peroxide. The reaction is catalyzed by a variety of compounds (table II) of which hexacyanoferrate(II) is perhaps the most suitable [64]. It gives a linear dynamic range over several decades. The luminol method is hampered by the high working pH (>10) and strong interference from reducing substances e.g. uric acid. This makes the method less suitable for clinical applications. A new group of reagents is the disubstituted oxalates which work at neutral pH [65]. Methods using such compounds give a somewhat lower sensitivity than those with luminol but seem to be less influenced by reducing substances.

The oxalates which give an efficient chemiluminiscence are insoluble in aqueous solutions and the organic solvents which have to be used introduces several new problems.

2.4 Catalytic disproportionation

The catalyzed disproportionation of hydrogen peroxide



has been the basis for two different detection principles for hydrogen peroxide, either measuring the oxygen formed or measuring the heat liberated in reaction (16).

2.4.1 Oxygen measurements

The oxygen formed in reaction (16) is reduced amperometrically at an ordinary membrane covered oxygen electrode [87]. Both catalase [74] and metal complexes [75] have been used as catalysts. Catalase is used either in soluble form [76] or immobilized onto the oxygen sensor [88]. These methods have the advantage of being insensitive to interferences from other electroreducible species since they are excluded by the gas permeable membrane.

On the other hand these methods are rather slow and they lack precision at low concentrations of hydrogen peroxide.

2.4.2 Thermometry

Monitoring the liberation of heat from chemical reactions is a well known analytical technique for quantitative measurements of enzymatically active compounds [88]. The temperature change obtained from reaction (16) is directly proportional to the number of moles of hydrogen peroxide reacted.

A column filled with platinized carbon [78] was used in a FIA system to fully decompose an injected hydrogen peroxide sample (paper III). The temperature change was sensed by a termistor placed at the outlet of the column [99]. The method is linear over a wide range but a long time for temperature equilibration is needed between each sample, especially for low concentrations of hydrogen peroxide.

3. FLOW ANALYSIS - FIA

3.1 Introduction

Samples can be introduced either continuously or as a well defined plug in a flowing stream for transport to a detection device where the concentration of the analyte is measured. The sample can be processed in a number of ways in the flow system (e.g. dilution, chemical reaction, and removal of interferences). The operations can be performed automatically and reproducibly as the sample is transported from the place of introduction to the place of detection. The carrier may be a homogeneous liquid, or it may be segmented by gas-bubbles [100]. The introduction of gas-bubbles decreases the dispersion of the sample drastically and consequently it decreases the time for an analysis. This technique is the most exploited and it is used in many commercial auto analyzer systems. A theoretical model which describes the dispersion profile of a sample in gas-segmented liquid carriers has been derived [101].

A newer concept of flow analysis based on the injection of a small sample volume into an unsegmented carrier stream was introduced in the mid seventies [102,103]. This technique (FIA), differs entirely from the continuous flow gas-segmented analysis since it works in a non-equilibrium mode, i.e. no steady-state conditions are achieved. Controlled dispersion is obtained through reproducible injection of sample into a constant flowing carrier stream and through reproducible timing. The sample zone is broadened during the transport to the detector due to dispersion. The sample will therefore become diluted and hence the sensitivity will be impaired. Peak broadening will also decrease sampling frequency. Although the broadening should be kept as low as possible high dilution may be introduced on purpose depending on the application.

In papers II, III and V a large dilution was required to reduce interferences and to accommodate the method to high concentrations of analyte. On the contrary a low dilution was used in papers IV and V to attain the sensitivity demanded.

Dispersion is a very complicated hydrodynamic process characterized by conventional and diffusional forces. Theoretical descriptions of this process have been the subject of many papers [104-106]. The models can be based either on the concept of axial-dispersion [107] or on the tank-in-series approach [108].

FIA has the advantage of being a simple, inexpensive and versatile technique, easy to construct and to adapt for different analytical purposes [109,110]. A FIA system comprises a peristaltic pump, an injection device and a detector connected by means of tubings with small internal diameter (typically 0.5 mm) (paper II, fig. 1). The system may be extended using more than one carrier or reagent line and inserting various kinds of components in the flow path. With such modifications chemical reactions, dialysis, titration, extraction, dilutions etc. can be performed [111]. In the present work flow-through components such as dialyzers, immobilized enzyme reactors, columns for removal of interferences (papers IV and V) and dilution coils (papers II and III) have been used.

3.2 Enzymes

There exist an overwhelmingly large number of methods utilizing enzymes for analytical purposes [88,96] with clinical or medical significance. The most important reasons for using enzyme mediated reactions in analytical chemistry are the selectivity and the high catalytic rate. The high selectivity is particularly important when complex sample mixtures (e.g. biological samples) are analyzed. Time consuming sample pretreatment steps can thus be avoided. The development of enzymatic methods in chemical analysis is, however, hampered by the fact that most enzymes are not easily accessible.

Immobilization of enzymes onto water insoluble matrices bears additional advantages in analytical chemistry. Expensive and rare enzymes may be reused for many analyses. The usable lifetime is determined by the stability of the immobilized enzyme. Increased stability has been reported for several enzymes when they are immobilized. Purification of the enzyme before immobilization or stabilization of the immobilized enzyme by intramolecular crosslinking have also been suggested in order to enhance the stability [88].

Another advantage obtained upon immobilization is that a large amount of enzyme can be used for each analysis. The time for an enzyme reaction to go to completion can thus be decreased to a few seconds compared to several minutes when normal concentrations of soluble enzymes are used. This is particularly useful in FIA systems when high sensitivity and high sample throughput are required (papers III, IV and V).

3.2.1 Enzyme reactors

Enzymes can either be immobilized directly onto the analytical sensor or immobilized in a reactor separated from the detection device. In the latter case it is possible to obtain optimal experimental conditions for the performance of both the enzyme and the detector as described by [112]. A special case where there is incompatibility in the optimal conditions for each enzyme in a coupled enzyme system is shown in papers IV and V.

Enzyme reactors are usually open tubular or packed bed. In open tubular reactors the enzyme is immobilized at the inner wall of a tubing whereas in

packed bed reactors the enzyme is immobilized onto small, incompressible porous particles. Due to the very large active surface available for immobilization, the latter is by far the most convenient reactor type for obtaining a high conversion efficiency. Complete conversion of substrate to product is normally a desirable property. High sensitivity along with less influence from e.g. pH, buffer ions, temperature or flow rate are the main benefits obtained with a high conversion efficiency [113]. Full conversion (>99.99%) of hydrogen peroxide to either an absorbing (paper III, IV) or an electroactive (paper V) species is accomplished in very small (20-180 μ l) enzyme reactors in FIA-systems. A small reactor volume is advantageous in order to minimize peak broadening. With enzymes of low inherent activity it may be problematic to achieve proper conditions for full conversion in a sufficiently small reactor volume. Conversion less than 100% may be used in this case rather than introducing very large reactors resulting in severe peak broadening.

The limited pressure range of peristaltic pumps has to be taken into account and special care has therefore to be observed when packed bed reactors are inserted into the flow path. Proper dimensioning of the reactor and the support material are important to avoid excessive pressure drops (paper IV).

3.2.1.1 Enzyme support and immobilization

High porosity and small pore sizes give large enzyme loadings [114]. The lower limit in pore diameter is set by the size of the enzyme. Particles of small size should be used for effective mass transfer and to obtain minimal dispersion in the reactor. Large pressure drops may however be an obstacle when very small particles are used.

Enzymes can be covalently attached to porous glass using a number of different immobilization techniques [115]. The technique chosen will depend on factors such as the chemical and physical properties of the enzyme as well as the operational conditions required for the analysis. The enzymes used in this work were immobilized onto porous glass by diazotlinkage [116]. Galactose oxidase (papers IV and V) and peroxidase (paper V) were immobilized onto an arylamino-activated glass by this procedure. Almost 100% of the enzymes were retained on the glass after immobilization. In paper IV

peroxidase was immobilized to a more hydrophilic support to prevent adsorption of products formed in the enzyme reactor.

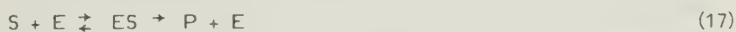
3.2.1.2 Enzyme purification and loading

A thorough purification of the enzyme prior to immobilization may be of paramount importance in order to get an effective enzyme reactor (papers IV and V). Commercial enzyme preparations usually contain impurities either remaining as a rest from the manufacturing process or added to the enzyme preparation as preservatives or stabilizers. These impurities e.g. proteins or small inorganic or organic compounds, may compete with the actual enzyme during the immobilization reaction resulting in a lower loading density. Small molecules are routinely removed from the enzyme preparation by ordinary dialysis whereas proteins are not. In severe cases the remaining protein part contains compounds e.g. enzymes which may act as interfering catalysts in the enzyme reactor. Catalase, a very common impurity in some enzyme preparations act as such in analytical applications where hydrogen peroxide is the species to be measured. Most of the catalase present in a preparation of galactose oxidase was effectively removed by purification with an ion-chromatography column (papers IV and V).

3.2.1.3 Kinetics in enzyme reactors

A measure of the catalytic activity of an enzyme reactor can be of importance when optimizing the analytical system [116].

Many complicated enzyme (E) systems may well be approximated by simple one substrate (S) Michaelis-Menten kinetics (eq(17))



as long as the cosubstrate(s) and the coenzyme are in excess.

The rate expression for eq(17) is given by

$$-\frac{dS}{dt} = \frac{V_{\max} \cdot S}{K_M + S} \quad (18)$$

where K_M is the Michaelis-Menten constant and $V_{\max}$ is the reaction rate

when $S \gg K_M$. In the analytical useful region i.e. when $S \ll K_M$ a first order rate expression is obtained

$$-\frac{dS}{dt} = K \cdot S \quad (19)$$

$$\text{where } K = \frac{V_{\max}}{K_M}$$

The same expression will hold for the enzyme reactor but the rate constant K may be lower due to restraint in mass transfer in the reactor.

Integration of equation (19) yields for a plug-flow enzyme reactor [111]

$$\frac{S}{S_0} = \exp(-K_{\text{app}} \cdot \tau) \quad (20)$$

where S_0 and S are the substrate concentrations at the inlet and outlet of the reactor respectively and τ is the mean residence time for the substrate in the reactor. K_{app} is the apparent first order rate coefficient of the reactor when the mass transfer limitation is taken into account.

Eq(20) was used in papers IV and V to estimate the enzyme activity of immobilized galactose oxidase reactors. Catalase, present as an impurity and inadvertently co-immobilized with the galactose oxidase, was found to significantly decrease the yield of hydrogen peroxide produced in the reactor, especially at low flow rates. To optimize the analytical system the decomposition of hydrogen peroxide was studied experimentally and a theoretical model based on two successive first order reactions was found to apply quite well to the experimental results (papers IV and V).

3.3 Interferences

3.3.1 Dialyzers

Dialyzers can be successfully used in FIA for on-line separation of low molecular weight sample components from larger molecules such as proteins [117] (papers IV and V). The active part of a dialyzer is a semi-permeable membrane which separates the sample injection line physically from the detector line in the FIA-system. The analyte dialyzes through the membrane whereas larger molecules are excluded. Since dialysis is a diffusion controlled process the amount dialyzed is ideally proportional to the

amount injected into the FIA-system as long as the operational conditions e.g. flow rates, temperature, pH etc are held constant. Factors such as size and geometry of the dialyzer and the properties of the membrane strongly affect the efficiency of the dialysis and should therefore be considered in each application. To achieve a high dialysis efficiency the membrane area should be large and the mean residence time long. The dialyzer may cause a large increase in dispersion in a FIA-system. The channels can be filled with solid glass beads to minimize this negative effect of the flow dialyzer (papers VI and V). The glass beads will disrupt the laminar flow [105] in the dialyzer and this was shown to be of profound importance when highly viscous samples e.g. serum samples were measured. The increased radial mixing obtained with the glass beads promotes the convective component of the mass transfer to the membrane surface. The dialysis factor becomes therefore less dependent on the diffusion coefficient in the solution.

Dialyzers may also be very convenient when a large dilution of the sample is required. With a dialyzer with a small membrane area a large dilution factor can be obtained without significantly increasing the dispersion of the injected sample (paper V).

3.3.2 Chemical treatment

In analysis of very complex matrices e.g. biological fluids, there is a risk of introducing interfering species even if certain precautions are taken (e.g. dialysis and enzyme reactors). Serum and urine contain reducing substances such as ascorbic acid, uric acid etc. and these compounds, not excluded by a dialysis step, interfere in a complex manner. They may act as substrates for the peroxidase and will thus compete with the analytical reagent for hydrogen peroxide. Choosing a suitable reagent of a sufficiently high concentration this interference may be eliminated. Another, more severe interference, is caused by the capability of these substances to reduce the products formed in the peroxidase catalyzed oxidation of the reagent by hydrogen peroxide [118]. The only way to eliminate this interference is to remove the reducing agents. Alkali de-proteinization [119] or oxidation of the reducing agents with heavy metal salts [120] are some of the methods which have been recommended for the elimination of these interfering species. On-line methods for sample

treatment in flow analysis have also been reported in the literature. Columns for either chemical [16] or electrochemical oxidation [15] of reducing substances have been used.

A cation exchanger column saturated with Cu(II)-ions was used to remove interferences from reducing agents in the analysis of serum and urine samples (papers IV, V).

3.4 Detectors

A large linear range, a high selectivity, a small peak broadening and a low detection limit are desirable for the detectors used in FIA. Those factors that control the concentration detection limit are of prime importance as well as those contributing to peak broadening [121].

In practice, efforts are directed to minimize the dispersion in the whole system and consequently the contribution to the total dispersion by the detector should be negligible. This means that the detection volume should be much less than the peak broadening and the response time should be short in relation to the signal rise time. Moreover, the dynamic character of FIA systems demands a large dynamic range of the detection device. Baseline drift is of less importance comparing to many other analytical systems since in FIA the signal is continuously measured in relation to the background.

3.4.1 Spectrophotometric detection

The spectrophotometric detectors commonly used in HPLC fulfill most of the requirements of an ideal detector for FIA. A detector volume of typically 10 μ l will not contribute significantly to the overall dispersion. The large linear dynamic range of a spectrophotometric detector is demonstrated in paper IV, (10 μ M to 14 mM galactose). The response for 14 mM galactose corresponds to an absorbance of 2, which is the upper limit of most spectrophotometers.

Apart from interferences originating from compounds which absorb at the same wavelength as the species to be measured, effects arising from refractive index changes may become significant when analysing very low

concentrations of the analyte. Such an effect is minimized by diluting the samples (paper IV) and using appropriate flow cell design [122].

3.4.2 Amperometric detection

Amperometric detection at solid electrodes has been widely used in HPLC [123] and has found an increasing use in FIA. Large dynamic ranges are obtained with amperometric detection and effective dead volumes less than 10 μl are easily attained [83] since the electrochemical process occurs at the surface of the electrode.

Although the detection response is a contribution of all the electroactive species reaching the electrode surface, selectivity can be improved by judicious selection of the redox potential.

Many useful designs of amperometric detectors in flow analysis are described in the literature [123-126] of which the tubular, the thin layer and the wall-jet are the main configurations.

Wall-jet detectors, in which the stream flows from a nozzle perpendicularly onto the electrode surface, exhibit high sensitivity with detection limits in the nM range due to a very effective mass transfer of sample molecules to the electrode surface. A dead volume in the order of 1 μl is readily obtained with this configuration [127], especially with an electrode of a small area (papers II, III).

The combination of the wall-jet electrode and a FIA system is very advantageous in reducing poisoning or adsorption effects. In FIA, the time of contact between the sample and the electrode is normally short and those species occasionally adsorbed at the electrode surface are washed away between injections (paper II).

Since amperometric flow through detectors are sensitive to variation in the flow rate, pulse free delivery systems should be used, although flow restrictors placed after the peristaltic pump are usually sufficient (papers II, III, V).

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DETERMINATION OF HYDROGEN PEROXIDE IN PICKLING BATHS FOR COPPER ALLOYS BY LINEAR SWEEP VOLTAMMETRY

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SUMMARY

Hydrogen peroxide in pickling baths for copper and copper alloys can be determined by linear sweep voltammetry with a glassy carbon electrode. The oxidation mechanism changes around 0.15 M H_2O_2 . Catalytic decomposition was found to be much smaller at glassy carbon electrodes than at platinum electrodes. An almost linear calibration curve was obtained up to 60 mM H_2O_2 . Interferences from Cu^{2+} , Zn^{2+} , Ni^{2+} , Al^{3+} , Fe^{3+} and Pb^{2+} as well as from the stabilizers were small. All measurements were made in sulphuric acid solutions.

The use of hydrogen peroxide in pickling baths has increased during the past few years, as it causes fewer environmental problems than some other oxidants, e.g. chromates. The hydrogen peroxide in the baths can usually be determined precisely by visual titration using permanganate. Nevertheless, there is a need for an instrumental method which can be used either manually or automatically at the site of the baths.

Pickling baths for copper and copper alloys, as well as baths for some other metals, contain 0.5–2 M sulphuric acid and 0.1–0.6 M stabilized hydrogen peroxide. The metal concentration will increase with use up to the limit set by the regeneration routine, usually 0.5 M for copper. The metal interferences considered in this work were from Cu^{2+} , Zn^{2+} , Ni^{2+} , Fe^{3+} , Al^{3+} and Pb^{2+} . Only a few of the known methods for the determination of hydrogen peroxide can be used in samples with the mentioned compositions. Direct electrochemical oxidation of hydrogen peroxide seemed to be the most promising one.

Harrar [1] developed a coulometric determination of hydrogen peroxide in which the oxidation was made at a platinum anode at +0.93 V vs. SCE. Guilbault and Lubrano [2] used an amperometric oxidative detection method for enzymatically produced hydrogen peroxide. A patent has also been granted for an electrochemical method for hydrogen peroxide determinations [3]. Very many papers on the electrochemical properties of hydrogen peroxide have been published. Hickling and Wilson [4] studied the anodic decomposition at Pt, Au, Ni and graphite electrodes. Lingane and Lingane [5] used chronopotentiometry in a mechanistic study of the oxida-

tion at a platinum anode in sulphuric or perchloric acid. The mechanisms of catalytic decomposition and oxidation at carbon electrodes have been studied recently by two research groups [6–10].

The overall reaction consumes 4 electrons and the final products are oxygen and water. There is also a non-electrolytic decomposition to oxygen and water and the electrode material will often catalyze this reaction. In the case of carbon electrodes, the carbon can be oxidized to carbon dioxide. The half-wave potential as well as the anodic current depend on the surface condition of the electrode.

EXPERIMENTAL

Voltammetric measurements

Voltammetric measurements were made with a polarograph (Princeton Applied Research, model 174A) and an x-y recorder (Houston 2000). The voltage was scanned in the positive direction from 950 mV to 1700 mV vs. Ag/AgCl at a rate of 200 mV s⁻¹. A more powerful potentiostat (80 mA output) was used in some experiments; the start potentials and scan rates were also varied as indicated in each case under Results.

Working electrodes were made from glassy carbon rods (3-mm diameter; Le Carbone Lorraine, Paris). Smaller electrodes made from glassy carbon of unspecified origin were also used. The carbon was either glued into a glass tube using an epoxy resin or enclosed with heat-shrinkable tubing (Alpha, USA, Fit-221). A commercial glassy carbon electrode (Princeton Applied Research) was also used. The electrodes made by the different methods had almost the same properties. The glassy carbon surface was polished with 1- and 0.25- μ m alumina (Struers, Copenhagen), cleaned in an ultrasonic bath, and oxidized at +1.7 V during 15 min in a solution containing 0.1 M H₂O₂ and 1 M H₂SO₄, which was stirred vigorously. The reference electrode was an Orion double-junction type (model 90-02). The outer chamber was filled with 1 M H₂SO₄. The auxiliary electrode was a platinum foil separated from the solution by a clay filter. The cell solution volume was 30 ml.

Three types of hydrogen peroxide solutions were used. Perhydrol (Merck, Cat. No. 7209) of p.a. quality and two moderately stabilized types for technical purposes, viz., HyBrite (FMC Corp., USA) and MS400 (Nord Nero AB, Sweden). A solution of an auxiliary stabilizer, MS402, was added in some cases. It contained no hydrogen peroxide, as it is intended for rejuvenation of baths or for producing special surface effects.

Hydrogen peroxide stock solutions were standardized by visual titration with permanganate standardized against oxalate. The procedures followed essentially those described by Kolthoff et al. [11].

RESULTS AND DISCUSSION

Exploratory studies

Ideally a method for use in the factory should be as direct as possible and the reading should be a direct measure of the required quantity, i.e. the hydrogen peroxide concentration. Voltammetric measurements were therefore made directly in the undiluted baths.

The catalytic decomposition of hydrogen peroxide interfered strongly at platinum electrodes. Bubbles of oxygen formed so rapidly that the peak trace became very noisy even at high sweep rates. In more dilute solutions, the condition of the electrode surface was important as noted by earlier investigators. Addition of stabilizer or metal ions also changed the peak current and the peak potential. The catalytic hydrogen peroxide decomposition was small on gold electrodes as has been observed before [12]. However, the overvoltage was larger and the wave was distorted. Moreover, the addition of stabilizer or metal ions again affected the response. Therefore gold seems to be unsuitable for the intended application.

The oxidation wave was well-behaved and much more reproducible on glassy carbon electrodes. Figure 1 shows calibration curves obtained with the three types of hydrogen peroxide. It can be seen that the slope is changed at around 0.15 M H_2O_2 . The calibration as well as the peak potential are fairly independent of the stabilizer below this concentration. Above 0.15 M H_2O_2 the kind and amount of stabilizer are important for the response. Above 0.15 M H_2O_2 , the peak potential increased with the concentration of hydrogen peroxide until the peak almost merged with the oxygen evolution wave. A high scan rate, 2 V s^{-1} , had to be used in order to avoid excessive interference from oxygen bubbles on the electrode.

The reaction is of the first order [10] with respect to the hydrogen peroxide concentration at carbon electrodes (in contrast to the half order [13] at platinum electrodes). The reaction proceeds in steps and the reacting species are weakly adsorbed at low concentrations but firmly adsorbed at high concentrations. In the solutions used in this work, there seem to exist at least two different mechanisms for the oxidation, and the second type dominates above 0.15 M H_2O_2 .

After a period of use in strong hydrogen peroxide solution, the glassy carbon surface becomes covered with a loose layer of carbon dust caused by pitting during the oxidation of carbon to carbon dioxide [9].

The results suggest that the oxidation current at glassy carbon electrodes should be fairly independent of the stabilizer and the metal content at a hydrogen peroxide concentration of 0.1 M or less. The peak potential, however, will still be dependent on the composition of the bath. A method for hydrogen peroxide determination should therefore involve a dilution to 0.1 M or less as a first step, and the peak current should be used in the evaluation.

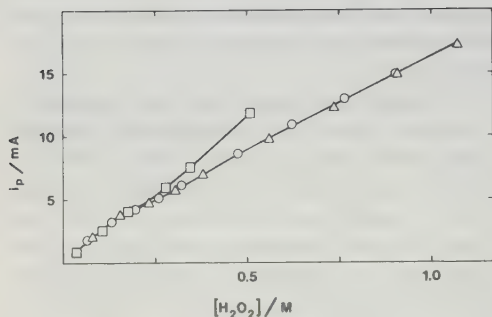


Fig. 1. Calibration curves for hydrogen peroxide at a glassy carbon electrode (diam. 1 mm). Sweep rate 2 V s^{-1} . ($\square$) Perhydrol; (Δ) HyBrite; ($\circ$) MS 400.

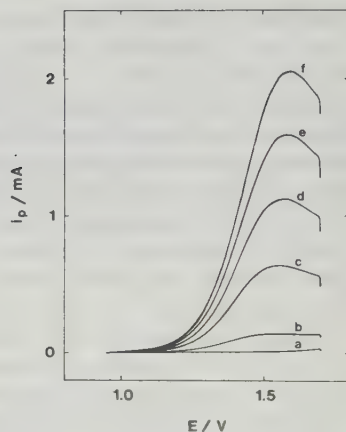


Fig. 2. Linear sweep voltammograms of hydrogen peroxide in $1 \text{ M H}_2\text{SO}_4$ at a glassy carbon electrode (Le Carbone). Sweep rate 200 mV s^{-1} . (a) Background; (b) 3.57 mM ; (c) 17.7 mM ; (d) 31.7 mM ; (e) 45.5 mM ; (f) $59.1 \text{ mM H}_2\text{O}_2$. The potential is measured vs. Ag/AgCl .

Method for determination of hydrogen peroxide

Figure 2 shows a series of voltammograms recorded in solutions of varying hydrogen peroxide concentration up to 0.06 M . The sweep rate affects both the shape and the position of the wave. It is easier to read the peak current at low sweep rates, especially if the hydrogen peroxide concentration is low. The pretreatment described in the Experimental section is essential for a reproducible wave; at an untreated glassy carbon electrode, the wave may not appear at all. Even after pretreatment there is a small decrease in peak current from the first to the following sweeps. A series of measurements were therefore started by three scans of the background in a solution without hydrogen peroxide; the value of the background current was taken from the third scan. Two scans were always made in sample solutions containing hydrogen peroxide and the peak current was measured from the second one. The solution was stirred vigorously between scans to remove oxygen bubbles from the electrode surface. The electrode surface should not be touched between measurements; polishing is not necessary even after extended use.

A typical calibration graph for HyBrite-stabilized hydrogen peroxide was linear over the range 4×10^{-3} – $6 \times 10^{-2} \text{ M}$ with peak currents ranging up to 2 mA , and passed through the origin. The other two types of hydrogen peroxide gave identical calibration curves. The peak can be observed down to about $2 \text{ mM H}_2\text{O}_2$. The detection limit is estimated to be $0.1 \text{ mM H}_2\text{O}_2$ if the peak potential is known from a calibration curve. The variations in background current and electrode condition during 8 h have been taken into

account in this estimation. The noise of the baseline itself is very low so that even lower detection limits could be obtained with a slightly modified procedure. The slope of the calibration curve will increase slightly (5%) with time for a new electrode until a steady state is obtained ($\pm 2\%$). However, there may sometimes be sudden larger changes (10%), especially when the electrode is used on real pickling baths in the factory. The electrode was therefore calibrated once a day.

The temperature coefficient of the peak current was $+1\% \text{ K}^{-1}$. All measurements reported in this paper were made at room temperature, $23 (\pm 1) ^\circ\text{C}$.

Interferences

Table 1 shows that the variation in peak current is small when the concentration of sulphuric acid is varied up to 2 M. If the baths are diluted with 1 M H_2SO_4 , variations in acid concentrations of the baths will be further levelled out. For actual pickling baths, the overall error from this source was estimated to be less than 1%. The error is always negative if calibration curves are made in 1 M H_2SO_4 .

Variations in the acid concentration have a rather large effect on the peak potential but this will not affect the results if the evaluation is made from the difference between the peak current and the background current at the same potential.

The interferences from metal solutions were studied in peroxide solutions containing 1 M H_2SO_4 to which the metal salts had been added in the form of sulphates. The peak current decreased 2% when Cu^{2+} , Zn^{2+} , Ni^{2+} , Al^{3+} or Fe^{3+} was added at the 50 mM level. The decrease in current was thus about the same for all the metals and was a linear function of the metal concentration. Considering the dilution which precedes the measurement, the interference from any of the metals mentioned as well as from a saturated solution of lead ions, should be negligible.

The slope of the calibration curve was the same for all the three peroxide solutions tested. If additional stabilizer is added, however, this may no longer be true. When the sample solutions contained 1% (v/v) MS402 as auxiliary stabilizer, the peak current for 60 mM H_2O_2 decreased 13%. The

TABLE 1

Variation of i_p (in mA) as a function of the sulphuric acid concentration for the P.A.R. glassy carbon electrode at a sweep rate of 0.5 V s^{-1}

[H_2O_2](mM)	[H_2SO_4](M)			
	0.3	1.0	2.0	3.0
17.6	4.28	4.42	4.12	3.75
52.5	12.2	12.9	12.1	11.5

decrease was linearly dependent on the concentration of the stabilizer. Thus if baths containing large amounts of stabilizer are to be analyzed, a separate calibration curve should be made in solutions to which the stabilizing agent has been added.

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AMPEROMETRIC DETERMINATION OF HYDROGEN PEROXIDE IN PICKLING BATHS FOR COPPER AND COPPER ALLOYS BY FLOW INJECTION ANALYSIS

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SUMMARY

The hydrogen peroxide is oxidized at +1.5 V vs. SCE at a glassy carbon electrode of the wall-jet type. The samples are diluted about 100 times in a dispersion coil before entering the amperometric detector. The calibration curve is linear from 10^{-4} to 1 M H_2O_2 , when 5- μl samples are used. With 50- μl samples the detection limit decreases to 10^{-6} M H_2O_2 . Neither metal ions (Cu^{2+} , Zn^{2+} , Ni^{2+} , Al^{3+}) up to 0.5 M nor changes in the sulfuric acid concentration of the samples between 0.1 and 1 M interfere with the hydrogen peroxide determination. About 75 samples can be analyzed per hour.

An automated method is needed for monitoring of hydrogen peroxide in pickling baths in the metal-working industry, because better control of bath composition ensures better quality of the finished product. Precise control will also prevent waste of chemicals and thus improve the process economy. Hydrogen peroxide has become used more extensively, mainly because of its environmental advantages over chromates. A typical bath for copper pickling contains 0.5–2 M sulfuric acid and 0.1–0.6 M highly stabilized hydrogen peroxide. The metal ion concentration increases with time up to approximately 0.5 M.

Few methods except electrochemical ones are well suited for the intended application. A patent has been granted [1] for an amperometric method based on a platinum electrode. A linear-sweep method with a glassy carbon electrode has previously been developed in this laboratory [2], but instrumental methods do not necessarily provide a sufficient degree of automation for industrial use. Even a simple manual operation such as dilution may require the involvement of laboratory personnel in the operation of the instrument. Flow injection analysis (f.i.a.) provides a simple means for complete automation [3] and this approach was therefore selected in developing an automatic analyzer for hydrogen peroxide in pickling baths.

EXPERIMENTAL

Equipment

Samples were introduced with a slide injector (Chromatronix; 0.8 mm i.d. channels, 5 μ l, Kel-F) into a stream of 1 M H_2SO_4 (Fig. 1). The carrier flow rate was normally 1.5 ml min^{-1} and the connecting tubings (0.9 mm i.d.) were made of teflon. The length recommended for the dilution coil (b) is 1.4 m (see below). A coil (0.3 mm i.d., 100 cm long) was inserted between the peristaltic pump (Gilson, Minipuls 2) and the detector in order to damp the flow pulsations.

The working electrode was made of glassy carbon (Le Carbone, Lorraine, type V.10); the exposed circular surface had a diameter of 0.6 mm. The glassy carbon was glued into a glass tube with an epoxy resin. The electrode was mounted horizontally in a Plexiglas cell so that it could be pushed towards the inlet nozzle. The distance between the electrode and the nozzle was not critical but was kept around 0.5 mm. Theory [4] predicts that the response of a wall jet electrode of this type should be rather insensitive to variations in the distance to the nozzle. The reference was a saturated calomel electrode (Radiometer, K701) and the auxiliary electrode was a platinum wire. A suction tube was mounted close to the cell wall to keep the liquid level constant. The working electrode was ground with a fine emery paper, polished with alumina 1 and 0.25 μm (Struers, Copenhagen); and cleaned in an ultrasonic bath. The electrode was pretreated by keeping it at +1.5 V in 20 mM H_2O_2 and 1 M H_2SO_4 until the current became constant. This pretreatment seemed to last indefinitely with the analytical procedure used. The electrode was kept at the desired potential all the time because a stabilization period of at least one hour was required every time the apparatus was switched off.

Measurements were made with a polarographic analyzer (PAR, model 174) and a strip-chart recorder. The working electrode potential was +1.50 V vs. SCE unless otherwise stated.

Solutions and samples

Standard solutions were made from hydrogen peroxide (Perhydrol, p.a.; Merck) and standardized by visual titration with potassium permanganate which had been standardized against oxalate. Samples were prepared from a technical stabilized hydrogen peroxide (Hy-Brite, FMC Corp., U.S.A.). The effects of an auxiliary stabilizer were evaluated by using MS402 (EKA, Bohus, Sweden). Pickling baths were obtained from Gränges Metallverken, Sweden, and the tests reported here were made on a bath with the composition 0.1 M Hy-Brite H_2O_2 , 0.65 M Cu^{2+} , 0.24 M Zn^{2+} and 2 mM Fe^{3+} .

RESULTS AND DISCUSSION

Optimization of the flow injection system

The samples were diluted in coil (b) (see Fig. 1) between the injector and the detector. Three lengths, 0.7, 1.4 and 2.8 m (0.9 mm i.d., coil diameter 26 mm) were tested. Figure 2 shows the peak shapes obtained with 5- μ l

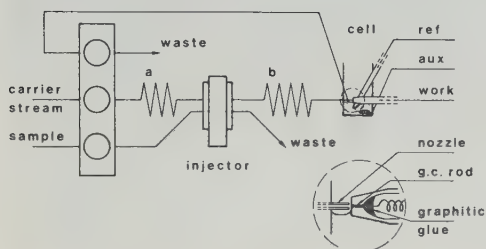


Fig. 1. Flow injection system for the determination of hydrogen peroxide in pickling baths. (a) Pulse flow damping coil; (b) dispersing coil. For further details, see text.

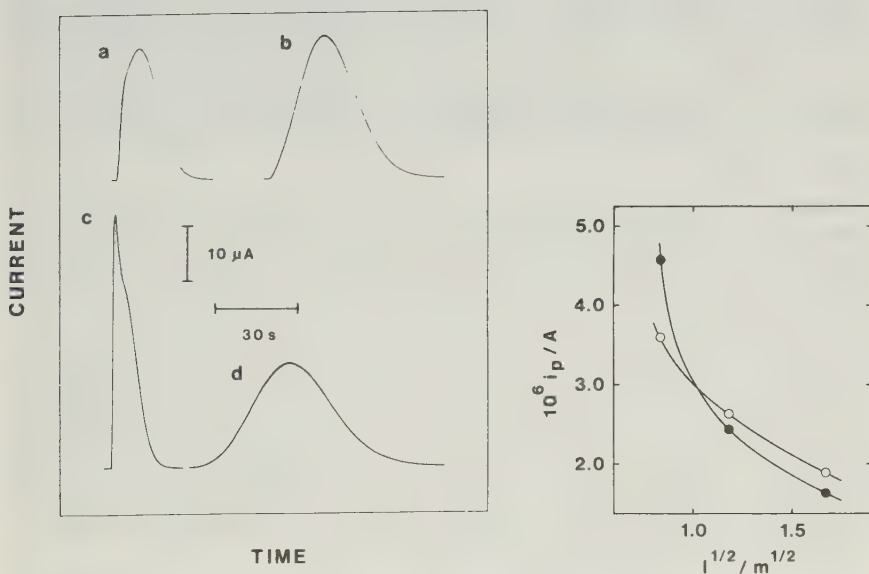


Fig. 2. Variation in peak shape when the length of the dispersing coil and the flow rate are changed: (a) 1.4 m, 2.19 ml min⁻¹; (b) 1.4 m, 1.06 ml min⁻¹; (c) 0.7 m, 2.19 ml min⁻¹; (d) 2.8 m, 1.06 ml min⁻¹.

Fig. 3. Variation of the peak height with the squared length of the dispersion coil. Samples were 0.1 M H₂O₂ in 1 M H₂SO₄. Flow rate: (○) 1.06 ml min⁻¹; (●) 2.19 ml min⁻¹.

samples. Comparison of peaks (a) and (b) shows that a high flow rate causes peak distortion, which becomes even worse with shorter coils (peak c). The peak shape improves (peak d) with longer coils and moderate flow rates. Dilution may produce double peaks because of the combined effect of Taylor and convective dispersion [5]; double peaks or severe distortion result in poor reproducibility of peak-height measurements. Small samples and a large inner diameter of the tubings are necessary for large dispersion, i.e., for high sample dilution in the flow stream. This high dilution and a reasonably high flow rate are required for proper detector performance. The remaining means to reduce peak distortion is to increase the tube length.

Figure 3 illustrates how the peak height varies with tube length for two different flow rates. The effect of changes in the flow rate is very small for the two larger coils because of the combined effects of dispersion and detector. After taking into account both the peak shape (Fig. 2) and the effect of flow rate and tube length (Fig. 3), a flow rate of 1.5 ml min^{-1} and a tube length of 1.4 m were selected. The 2.8 m coil could, of course, also have been chosen at the expense of a somewhat lower sample throughput and a slightly reduced sensitivity.

Electrochemical detection

Hydrogen peroxide can be oxidized electrochemically to oxygen and hydrogen ions [6, 7]. It also decomposes catalytically to oxygen and water in a non-electrolytic step. Carbon, if used as electrode material, can be oxidized to carbon dioxide either chemically by hydrogen peroxide or electrochemically at high potentials [8]. Reduction of hydrogen peroxide as a basis for an analytical method is out of the question for samples containing reducible metal ions.

Platinum catalyzes the non-electrolytic decomposition of hydrogen peroxide very efficiently even in the presence of high concentrations of technical stabilizers. This side-reaction contributes to oxygen bubble interference at the electrode. Gold does not cause any significant catalytic decomposition in solutions containing sulfate ions, but the oxidation is more irreversible and requires a more positive voltage. The oxidation is partly under kinetic control for all electrode materials and the reproducibility of the surface state is poor for platinum and even worse for gold. The surface properties of glassy carbon electrodes are reproducible when the electrode has attained a steady state [2]. Sample dilution improves the precision by reducing the surface oxidation of the electrode material. Because of a changed oxidation mechanism above $0.15 \text{ M H}_2\text{O}_2$, dilution is also necessary to obtain linear calibration curves.

Figure 4 shows the steady-state total current, the background current and the net current of the amperometric detector based on a glassy carbon electrode. The background current increases around $+1.4 \text{ V}$ and becomes a significant fraction of the total current at higher potentials. The relation between the total current and the background current varies with the sample

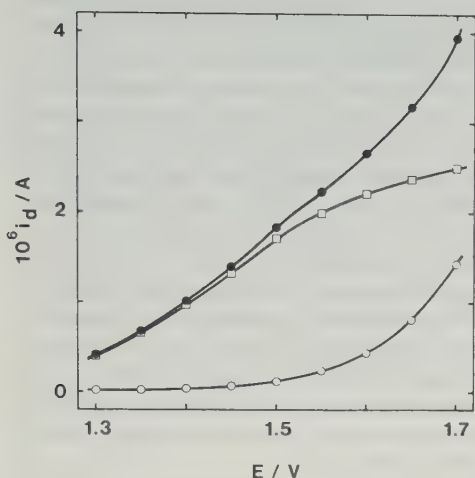


Fig. 4. Working electrode current (i_d) plotted versus the applied potential (E) measured vs. SCE, at a flow rate of 1.5 ml min^{-1} . ($\bullet$) Total current with a steady supply of $1 \text{ mM H}_2\text{O}_2$ in $1 \text{ M H}_2\text{SO}_4$; ($\circ$) background current obtained with $1 \text{ M H}_2\text{SO}_4$; ($\square$) net current after subtraction of the background.

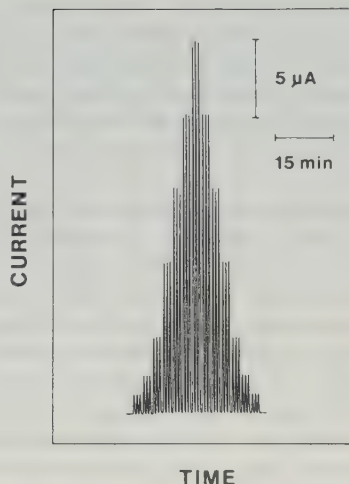


Fig. 5. Calibration record for triplicate injections ($5 \mu\text{l}$) in each direction of samples containing 0.050 , 0.100 , 0.200 , 0.400 , 0.600 , 0.800 and $1.00 \text{ M H}_2\text{O}_2$ in $1 \text{ M H}_2\text{SO}_4$. Flow rate 1.5 ml min^{-1} .

composition. The conditions in Fig. 4 correspond to the peak of an injected sample with a concentration of $0.1 \text{ M H}_2\text{O}_2$. A potential of $+1.5 \text{ V}$ was selected because it provides good sensitivity and tolerable background current.

The response of the detector depends on the rate of mass transfer, i.e., flow rate, geometry and viscosity. Doubling the flow rate increased the response by 11%, which is less than the increase expected from theory [4]. When the viscosity was varied, by varying the sulfuric acid concentration between 0.1 and 1 M , the response followed theory, i.e., it decreased with the viscosity to the power of -1.08 . The fact that the detector shows less flow dependence than expected, but still follows theory when the viscosity is varied, may be explained by a partial kinetic control of the oxidation current. The net current in Fig. 4 does not reach a plateau, which supports this explanation.

Oxygen bubble formation constitutes the main experimental obstacle in making measurements in hydrogen peroxide solutions. The bubbles block part of the electrode surface and interfere with mass transfer. Thus, signals are excessively noisy at higher concentrations of hydrogen peroxide. The difficulties can be largely overcome by sample dilution and an elec-

trode arrangement in which a rapid liquid flow displaces the bubbles, but the selection of surrounding materials is also of importance. Teflon should be avoided because the oxygen bubbles grow and accumulate on the surface, and break away causing disruption of the steady-state mass transfer. Very fast measurements are less affected as only small amounts of oxygen are produced. The use of pulse or fast sweep methods [2] with vigorous stirring between the sweeps or pulses, reduces the interference from oxygen bubbles; but the background currents were large and less well defined, restricting the range to relatively concentrated peroxide solutions. Under these conditions the peak position will vary with sample composition and surface state of the electrode. With the wall-jet electrode used in this work, bubbles are removed much more efficiently, and measurements can be made amperometrically at the constant potential. The background current then becomes much lower and more stable so that the range can be extended downward. The samples can therefore be diluted more, which further improves electrode stability and decreases interferences. The f.i.a. method provides another advantage, i.e., that the background current can be compensated automatically if peak height over the base line is measured.

Hydrogen peroxide determination

The amperometric detector was used for hydrogen peroxide determination using the flow injection scheme of Fig. 1. The 5- μ l samples were diluted about 100 times with 1 M H_2SO_4 in the 1.4-m dispersion coil. A typical calibration record is shown in Fig. 5. A strictly linear calibration curve ($r = 0.99997$) was obtained from the detection limit, 2×10^{-5} M, to 1 M H_2O_2 . The repeatability of successive measurements was better than 0.5% and the relative precision for repeated measurements during one day was 1% for the upper part of the range. The accuracy of the determinations was better than 0.5% when measurements and standardizations were done in rapid succession. The response was equal for samples made from p.a. hydrogen peroxide and from the technical product.

There was an increasing negative deviation when the sample concentrations were above 1 M H_2O_2 . This is due both to the IR drop in the solution and to partial oxygen coverage of the electrode. The range can of course be adjusted upward by increasing the size of the dilution coil or by decreasing the sample volume. The use of 1- μ l samples increased the linear response to 5 M H_2O_2 . The range can be adjusted downwards most simply by increasing the sample volume, which will decrease the dispersion and provide less dilution. A detection limit of 10^{-6} M H_2O_2 was obtained with 50- μ l samples introduced by a pneumatic loop injector. The peak current at the detection limit was 0.5 nA and the drift in the background current was 1.5 nA h^{-1} . The background current and the drift can be reduced by selecting a lower electrode potential.

Interferences

Variations in the acidity (0.1–1 M H_2SO_4) or metal ion content (0–0.5 M Cu^{2+} , Zn^{2+} , Ni^{2+} or Al^{3+}) of the sample did not affect the peak height. Increasing the acidity to 3 M H_2SO_4 reduced the peak height by about 1%. Iron(III) did not interfere as such, but the hydrogen peroxide decomposed very quickly in the presence of 0.5 M Fe^{3+} . Addition of extra stabilizer, up to 5% v/v, did not affect the peak height.

Standard additions were made to samples taken from an alloy pickling line. Correct recoveries, within 1% of calculated values, were obtained. Thus, hydrogen peroxide can be determined in pickling baths of the specified composition without interference. The errors produced by higher amounts of peroxide are small and can be eliminated by reducing the sample size.

Several advantageous circumstances contribute to the absence of interferences. Samples with high acidity or high metal ion concentration produced changes in the shape of the rising part of the f.i.a. peak, because the changed viscosity altered the dispersion profile. The peak height was not affected, however, and the viscosity changes therefore had no influence on the analytical signal.

The selected carrier electrolyte, 1 M H_2SO_4 , was used because it matched most of the samples with respect to viscosity. Less concentrated acid, 0.1 M H_2SO_4 , resulted in negative interference from metal ions, whereas 3 M H_2SO_4 , attacked the electrode chemically, so that the sensitivity of the electrode decreased with time.

Most of the improvements compared with the earlier method [2] are due to the greater dilution and the physical arrangement of the detector. The short exposure of the electrode surface to the sample is also of importance for the suppression of interferences from the stabilizer. The signal decreased with time when a solution containing a high concentration of peroxide and stabilizer was fed continuously to the detector. The height of the initial rise, however, was the same whether or not stabilizer was present. This behaviour can be explained by assuming that the stabilizer is adsorbed slowly at the electrode surface. In the f.i.a. method, the peak passes so quickly that the adsorption is slight, and desorption of the stabilizer is fast enough to ensure that a clean electrode is available for the next sample.

About 75 samples can be analyzed per hour which is much more than needed for the present purpose. Further development, based on building a dedicated instrument with pneumatic samplers, seems to be straightforward and is under way in this laboratory.

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DETERMINATION OF HYDROGEN PEROXIDE FOR APPLICATION IN AEROBIC CELL SYSTEMS OXYGENATED VIA HYDROGEN PEROXIDE

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SUMMARY

An amperometric method is described for the determination of hydrogen peroxide in reaction media used for production of dihydroxyacetone from glycerol by immobilized bacteria. The H_2O_2 was oxidized at +1.2 V vs. SCE at a glassy carbon flow-through electrode after dilution in a flow injection analysis system. Unexpected losses of H_2O_2 response in cell-free media necessitated a study of the cause. To validate the measurements two other methods for H_2O_2 determinations were used in parallel, a catalytic calorimetric method and an enzymatic flow injection method based on the use of immobilized peroxidase to produce a coloured product which was monitored spectrophotometrically. The three methods gave the same results. It was concluded that the responses of the methods were correct and that the loss of H_2O_2 is real and due to reactions with dihydroxyacetone or some of its decomposition products. A reverse reaction was also observed, i.e., the production of H_2O_2 from oxygen and decomposition products of dihydroxyacetone in an originally peroxide-free solution.

A suitable supply of oxygen is of the utmost importance for the productivity of aerobic cell systems, and the demands on the supply system become especially high in fermentors with high cell densities [1]. The solubility of oxygen in aqueous solutions is low compared to the normal substrate concentrations and the oxygen activity at the reaction site may be lowered still more by a slow mass transfer in the solution or within the cells. Enzymes such as catalase, present within the cell or in the cell membrane, can produce oxygen in situ through decomposition of hydrogen peroxide. Thus, addition of hydrogen peroxide to the medium is potentially a more efficient method of oxygenation than gas sparging. High concentrations of hydrogen peroxide may damage the cells, especially during the growth phase [2, 3], or may decrease the reaction rate by inhibition of enzymes. Increased productivity with hydrogen peroxide as the oxygen source has been demonstrated for both immobilized enzymes [4] and for resting immobilized cells [5].

Gluconobacter oxydans immobilized in beads of calcium alginate can be used for the production of dihydroxyacetone from glycerol [5]. The immobilized cells are alive but do not multiply during production of dihydroxyacetone and they are therefore more tolerant towards hydrogen peroxide than cells in the growth phase. The system is thus well suited for a methodical study of oxygenation through additions of hydrogen peroxide. A proper control of the peroxide concentration is nevertheless essential for high productivity without undue cell damage or inhibition.

This work is concerned with the development of methods for monitoring the hydrogen peroxide in the reactor with an electrochemical method very similar to that which has been used in earlier work on hydrogen peroxide determinations in pickling baths [6]. Because of an unexpected time dependence, it was compared to two other methods, each based on a different principle, in order to achieve mutual validation and assessment.

EXPERIMENTAL

Amperometric flow injection analysis

A 5- μ l sample was introduced into an unsegmented deaerated (vacuum) carrier stream of 0.2 M sodium acetate, pH 5.0, using a pneumatic injector. The sample was diluted 100 times in a dispersion coil and oxidized at a glassy carbon electrode, kept at +1.2 V vs. SCE by potentiostatic control. The electrode reaction is oxidation of hydrogen peroxide to oxygen and hydrogen ions, and the current is proportional to the concentration over a wide range. A new electrode is pretreated by keeping it at +1.2 V in a 10 mM peroxide solution until a stable value is obtained (some hours). After that, the electrode remains stable indefinitely if the potentiostat remains active. Further details of the method are available in an earlier paper [6].

Enzymatic spectrophotometric method

The flow injection system is outlined in Fig. 1.

A 25- μ l sample was introduced into a stream consisting of deaerated 0.1 M sodium phosphate, pH 6.0, by means of a pneumatically activated loop injector (Cheminert SVA 8031). Hydrogen peroxide from the sample oxidizes the immobilized peroxidase in the enzyme reactor and the peroxidase is then reduced during an oxidative coupling of 4-aminoantipyrine to 2,4-dichlorophenolsulphonate (DCPS). The coloured product is monitored at 514 nm in a 10- μ l flow cell (LKB model 2151). The flow rates were 0.70 and 0.35 ml min⁻¹ in the buffer and reagent lines, respectively.

Horse-radish peroxidase (EC 1.11.1.7, Sigma Type VI, Cat. No. 6140) was azo-immobilized [7, 8] onto porous glass (CPG-10, pore diameter 33 nm, 120–200 mesh; Electro Nucleonics, Fairfield, NJ). The enzyme-treated glass was packed into a reactor consisting of teflon tubing (1.0 mm i.d., 28 mm long, volume 22 μ l) connected to the detector with 0.3-mm i.d. teflon tubing. Mixing between reagent (10 mM DCPS, 1 mM 4-aminoantipyrine in

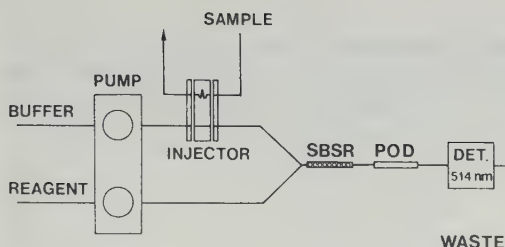


Fig. 1. Flow injection system for enzymatic determination of hydrogen peroxide. POD, peroxidase reactor; SBSR, single bead-string reactor.

0.1 M phosphate buffer, pH 6.0) and sample took place in a bead-string reactor [9] (i.d. 0.8 mm, length 100 mm, 0.5-mm diameter glass beads) which was inserted between the confluence point and the enzyme reactor. The samples were manually diluted 100 times before injection, to reduce the influence of substrate and products on the enzymatic reaction. The diluted samples were drawn into the sample loop by a separate peristaltic pump. The flow injection method was developed earlier at this laboratory for the determination of dissolved oxygen using other chromogens [10]. A thorough investigation of the oxidative coupling is under way.

Flow-through calorimetry

A 0.3-ml sample was introduced manually by a 4-way valve (Rheodyne) into a deaerated buffer stream consisting of 50 mM sodium succinate and 0.54 M glycerol, pH 5.0. Hydrogen peroxide from the sample was decomposed catalytically on platinized carbon (0.22 g) in a teflon reactor (i.d. 7 mm, length 18 mm) provided with a thermistor for temperature measurements [11]. A peristaltic pump (Bifok, FIA 08) was used to deliver the carrier solution (1 ml min⁻¹).

The reactor packing material was made by soaking granules of acid-washed carbon in hexachloroplatinic acid and drying it in an oven at 105°C for 1–2 h. The chloroplatinate was reduced at 300°C in a hydrogen stream until no acid fumes could be detected at the outlet. The amount of platinum was calculated to be 0.25 g g⁻¹ of carbon. The calorimetric method has been described in detail elsewhere [12].

Chemicals

Hydrogen peroxide was obtained from Merck (Perhydrol, 30%). Stock solutions were standardized by titration with permanganate which in turn had been standardized against oxalate. A 5.54 M stock solution of dihydroxyacetone (98% pure; Merck) was prepared well in advance to ensure complete monomerization [13]. Methylglyoxal (grade II, 40% aqueous solution; Sigma) and DL-glyceraldehyde (>97%; Fluka), hexachloroplatinic(IV) acid (10%, p.a.; Merck), activated carbon (granules, 0.5–1.0 mm; KEBO AB,

Sweden) and 4-amino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (4-aminoantipyrine; BDH Chemicals) were used as received. DCPS was prepared as described by Barham and Trinder [14]. All other chemicals were from commercial sources and of p.a. quality.

RESULTS AND DISCUSSION

Amperometric method

The calibration graphs for hydrogen peroxide were strictly linear from 10^{-4} to 10^{-1} M. Electrochemical oxidation of glycerol, dihydroxyacetone or the buffer components takes place to some extent (see Table 1). The resulting currents were so small, however, that the interferences should be negligible in most cases (<6% for samples taken under the normal process conditions of 1–10 mM H_2O_2 , 0–1.1 M dihydroxyacetone, 1.1–0 M glycerol, pH 5.0).

The effect of dihydroxyacetone on the electrochemical oxidation of hydrogen peroxide was investigated by cyclic voltammetry (Fig. 2). It can be seen that increasing amounts of dihydroxyacetone affect both the peak shape and the peak position. The actual concentration of dihydroxyacetone at the glassy carbon electrode will be at most 11 mM because of the dilution in the dispersion coil. Thus its effect on the response to hydrogen peroxide can be neglected. The same conclusion was found to hold for additions of glycerol.

Reactions of dihydroxyacetone

Dihydroxyacetone mutually isomerizes with DL-glyceraldehyde; both isomers can react in irreversible, acid-base catalyzed steps to form methylglyoxal, which in turn may react slowly to form, e.g., lactic acid [15, 16]. The isomerization is catalyzed by base, or possibly acid/base [17], and is

TABLE 1

Peak currents from the oxidation of sample components in the amperometric flow cell (The interference level is the H_2O_2 sample concentration which gives the same peak current as the compound)

Compound	Conc. (M)	Current (nA)	Interference level (mM H_2O_2)
Dihydroxyacetone	1.1	3.5	0.052
	0.5	1.8	0.027
	0.25	1.0	0.015
Succinate	0.05	0.32	0.004
Glycerol	1.1	0.49	0.007
	0.54	0.18	0.003
	0.27	0.13	0.002
Methylglyoxal	0.002	<0.05	<0.0007
Glyceraldehyde	0.011	0.43	0.004

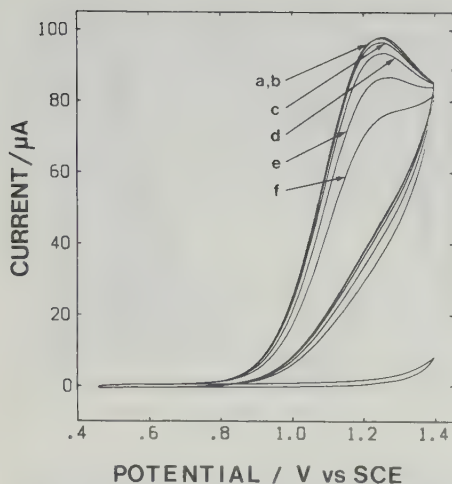


Fig. 2. Cyclic voltammograms of 5 mM H_2O_2 in (a) 0; (b) 0.011; (c) 0.039; (d) 0.093; (e) 0.22; (f) 0.47 M dihydroxyacetone. All solutions were 0.1 M in succinate, pH 5.0. The background current is shown as the bottom plot. Starting potential 0.50 V; sweep rate 50 mV s^{-1} .

quite rapid at higher pH. The formation of methylglyoxal is slow in water and in buffers like glutamate, succinate and pyrophosphate. Other ions, especially phytate and phosphate, act as powerful catalysts [18]. Methylglyoxal can be formed both from dihydroxyacetone and from glyceraldehyde although the latter reaction is slower. Some rate constants are available at 40°C [18] and at 50°C [15] but the data are not sufficient for a calculation of the rate of either formation of methylglyoxal or isomerization during measurement or production of dihydroxyacetone. It may be mentioned, however, that if Riddle and Lorenz's data [18] are applied to a solution containing 1.1 M dihydroxyacetone, 50 mM phosphate, pH 7.4 at 40°C , an initial production rate of methylglyoxal of $65 \times 10^{-3} \text{ moles l}^{-1} \text{ h}^{-1}$ is predicted.

Dihydroxyacetone (1.1 M) was mixed with 10 mM hydrogen peroxide and the mixture was analyzed electrochemically at successive times (Fig. 3). The response decreases with time at first, then it levels out to form a plateau which decreases by 4% during the first hour. Standards run before and after showed that the electrode response remained constant. The initial decrease of response was very fast at pH 5 and 6, but slower in more acidic solutions. The initial decrease of response was also noted at pH 7 and 9, but no clear plateau could be seen. The overall loss of response was very fast at pH 7 with only 4% of the initial amount remaining after 90 min. About 20% remained after the same time in a solution which had a pH of 9. The response decreased with the same percentage and the same relative time course, independently of concentration in the range 1–10 mM hydrogen peroxide. Standard additions produced a step followed by a rapid 10–15% decrease

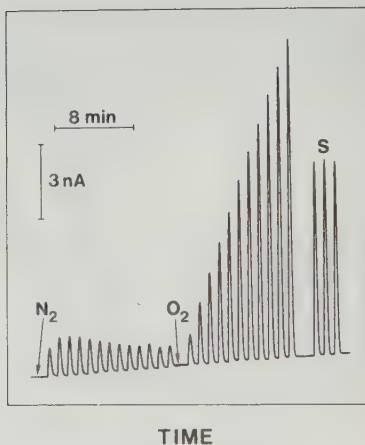
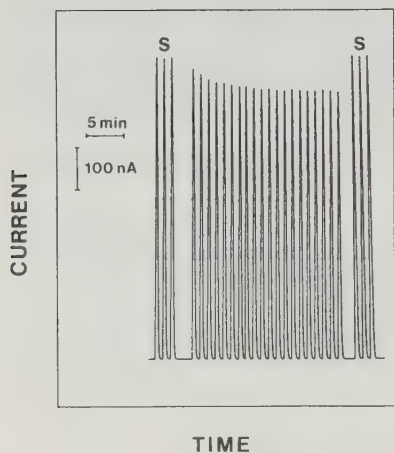


Fig. 3. The time-dependent decrease of the H_2O_2 signal in a solution containing 10 mM H_2O_2 and 1.1 M dihydroxyacetone in 50 mM phosphate buffer, pH 4.0. The S peaks are standards (10 mM H_2O_2) run before and after.

Fig. 4. The effect of oxygen on the peak signal (in 0.11 M dihydroxyacetone in a 0.1 M phosphate buffer, pH 7.0). S is a 0.1 mM H_2O_2 standard.

with the same relative shape as observed initially ($\text{pH} \leq 6.0$).

The reported behaviour indicates that 10–15% of the hydrogen peroxide is consumed in a side reaction or bound as an adduct or complex which is electrochemically inactive. The possibility that other methods might respond in a different way to this fraction necessitated a comparative study as well as a study of the reactions between hydrogen peroxide and other compounds which are likely to be present in the medium.

About half of the hydrogen peroxide added to a 0.28 M glyceraldehyde solution (pH 4.0) had disappeared after 20 min. The decrease in response was almost exponential. The reaction is thus fairly fast but the formation of aldehyde through isomerization is slow in acidic solutions [15]. The total effect on the peroxide consumption in a dihydroxyacetone solution should therefore be small. There were no other significant reactions except between hydrogen peroxide and methylglyoxal.

Reactions of methylglyoxal

The data given by Riddle and Lorenz [18] show that no methylglyoxal should be produced in the reaction medium at pH 5.0 but that some should be produced in a phosphate buffer at pH 7.0 as mentioned above. Besides the methylglyoxal produced in this way some might be added with dihydroxyacetone as an impurity. It is therefore important to study the interaction between methylglyoxal and hydrogen peroxide.

Hydrogen peroxide was added to a 2.3 mM methylglyoxal solution and samples were analyzed by the electrochemical method at successive times. There was no initial rapid decrease in response such as that described above. There was, however, a slow continuous decrease in response which amounted to about 10% after 30 min at both pH 5 and 7 in a 10 mM peroxide solution. The decrease was slightly faster in a 1 mM peroxide solution. These observations show that it is unlikely that methylglyoxal is involved in the initial rapid decrease of response in the dihydroxyacetone solution.

Reactions of oxygen in dihydroxyacetone solutions

A fresh dihydroxyacetone solution, prepared from deaerated buffer, was bubbled with nitrogen and sampled into the flow injection system. Figure 4 shows that small peaks with time-dependent amplitudes were produced. Part of the response is due to a direct electrochemical oxidation of dihydroxyacetone and the rest to a reaction involving oxygen. Even during the nitrogen bubbling, some oxygen is present from contamination during preparation. When the nitrogen was replaced by oxygen, the analytical system showed successively increased responses. On returning to nitrogen bubbling, the peak heights decreased slowly with time. When the experiment was repeated with a 2.5 mM methylglyoxal solution no influence of oxygen could be seen.

The conclusion to be drawn is that hydrogen peroxide, or a compound with identical effect on the amperometric response, is formed in a reaction involving oxygen. The reaction seems to be reversible as evidenced by the decrease in peak heights during the nitrogen bubbling. It is known that methylglyoxal is produced from dihydroxyacetone via intermediates [17]. A possible explanation is therefore that one of the intermediates forms an adduct with oxygen, which hydrolyzes to hydrogen peroxide and pyruvate or lactate. The presence of small amounts of pyruvate was indeed demonstrated by its reaction with NADH and lactate dehydrogenase. The reaction rate with oxygen at a constant partial pressure and excess of dihydroxyacetone is expected to be constant in agreement with the observed linear increase of the peaks in Fig. 4. Table 2 gives the pH and medium dependence of the reaction, which was found to behave in the same way and to be catalyzed by the same ions as the reactions studied by Riddle and Lorenz [18]. In fact, the rates agreed with those extrapolated from the work of Riddle and Lorenz to within a factor of two. All this leads to the conclusion that hydrogen peroxide is produced spontaneously in the reaction medium if oxygen is present.

Comparison between the electrochemical, enzymatic and calorimetric methods

The amperometric method behaves almost ideally and can be used for almost interference-free determinations of hydrogen peroxide except for one possible deviation i.e., the time-dependent decrease of response in the pres-

TABLE 2

Effect of pH and anions on the production rate of H_2O_2 in a 56 mM dihydroxyacetone solution continuously sparged with oxygen

Medium ^a	pH	H_2O_2 formation (10^{-6} M min ⁻¹)
Water	—	0
Phosphate	2.0	0
Succinate	5.0	0
Phosphate	5.0	0
Pyrophosphate	6.0	0
Phosphate	6.0	1.5
Phosphate	7.0	7.8
Phosphate	8.0	20
Pyrophosphate	9.0	7.2

^a0.1 M anion.

ence of dihydroxyacetone. It is therefore of interest to investigate whether the observed decrease in response reflects a real decrease in peroxide concentration or if it is an artefact of the method. Because both the enzymatic and the calorimetric methods were available in this laboratory and each measures hydrogen peroxide in a completely different way, it was decided to make measurements in parallel with the three methods.

Table 3 shows a comparison of the amperometric and calorimetric methods when they were used to follow the reaction in a solution containing dihydroxyacetone and hydrogen peroxide. It can be seen that the response decreases to the same degree with both methods. Table 4 gives a comparison of the amperometric and enzymatic methods when both were used to determine the peroxide in a series of solutions containing various amounts of dihydroxyacetone. Again, it can be seen that both methods record a decrease in the peroxide concentration as the dihydroxyacetone concentration increases. Similar results, with agreement between the responses of the two methods, were obtained at other pH values. Both dihydroxyacetone and glycerol produce transients in the spectrophotometer because of changes in the refractive index and this makes the evaluation somewhat more uncertain at low peroxide concentrations.

The enzymatic method was also used to check the mentioned production of peroxide in dihydroxyacetone solutions in the presence of oxygen. The results from the two methods paralleled each other very closely. The rates of peroxide production were found to be 7.8 and 8.1 μM peroxide min⁻¹ with the enzymatic and amperometric methods, respectively. The rates relate to 50 mM dihydroxyacetone, 0.1 M phosphate buffer, pH 7.0. The agreement between the results obtained by the three methods supports the conclusion that each method measures the true hydrogen peroxide concentration even in ageing dihydroxyacetone solutions.

TABLE 3

Comparison of the amperometric and calorimetric methods used to follow the decrease of the H_2O_2 signal with time in a solution containing 10 mM H_2O_2 and 0.55 M dihydroxyacetone in 0.1 M sodium phosphate, pH 7.0

Time of reaction (min)	Relative peak signal (%)	
	Amperometric	Calorimetric
0	100	100
2.5	78	76
6.4	65	65
10.3	55	55

TABLE 4

Concentration of H_2O_2 after 4.0-h incubation as measured by the amperometric and enzymatic methods

(Measurements were made in parallel in solutions initially containing 10 mM H_2O_2 and various amounts of dihydroxyacetone (DHA) in a 0.1 M pyrophosphate buffer, pH 9.0)

DHA conc. (M)	Relative peak signal (%)	
	Amperometric	Enzymatic
0	100	100
0.11	86	89
0.55	40	42
1.1	19	20

If some of the hydrogen peroxide reacts to form organic peroxides, the methods must either be rather insensitive to such peroxides or respond to the same extent. The rate constants for the reaction between some peroxides and peroxidase for the formation of "Compound I" have been evaluated [19] and were found to decrease by one order of magnitude between hydrogen peroxide and methyl peroxide. The rate decreased by another factor of ten for butyl peroxide and still more for branched chain compounds. Peroxidase is known to be completely insensitive to dialkyl peroxides whereas acyl peroxides may react more quickly than hydrogen peroxide. Reactions between hydrogen peroxide and aldehydes are known to produce mono- and di-hydroxyalkyl peroxides. The dihydroxydialkyl peroxides are unstable and decompose rapidly in alkaline media [20].

Even if there is an excess of capacity in the peroxidase reactor of the enzymatic method, it will not be sufficient to give the same response towards organic peroxides with rate constants which are orders of magnitude less than that for hydrogen peroxide. The calorimetric reactor also has excess of capacity but it is insufficient for decomposition of organic peroxides with low reaction rates. Furthermore, there might be differences between the heat produced by decomposing hydrogen peroxide and organic peroxides.

In the amperometric method, the potential of the glassy carbon electrode was selected on the rising part of the oxidation curve in a potential-current diagram in order to reduce the background current [6]. The response will thus be very sensitive to small changes in the oxidation potential of the compounds. The method as designed is thus expected to give either quite different responses to hydrogen peroxide and organic peroxides or to give no response at all to the latter.

Peroxides which equilibrate fairly fast with hydrogen peroxide, and which could therefore be measured via hydrogen peroxide can also be excluded because of the different residence times in the reaction zones. The mean residence times were 40 ms, 1.6 s and 10 s for the amperometric, enzymatic and calorimetric methods, respectively. Unless the equilibration takes <40 ms, the methods should have given different results.

Taken together, these arguments show that it is very unlikely that the response is due to organic peroxides to any significant extent. The electrochemical method described here is therefore well suited for determination of hydrogen peroxide in the biotechnical process mentioned. It is fast and accurate and can easily be adapted to automated sampling. Work is now in progress to use it for computerized control of the hydrogen peroxide concentration in this process.

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GALACTOSE DETERMINATION IN AN AUTOMATED FLOW INJECTION SYSTEM CONTAINING ENZYME REACTORS AND AN ON-LINE DIALYSER

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SUMMARY

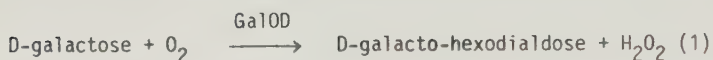
D-Galactose is oxidized in a reactor containing immobilized galactose oxidase to give hydrogen peroxide, which is determined spectrophotometrically after an oxidative colour-forming reaction in a peroxidase reactor. On-line treatment in a dialyzer, a metal-chelate column and a catalase reactor make the method essentially free from interferences, for e.g. serum samples. The effect of catalase impurities in the galactose oxidase enzyme preparation is illustrated. The linear range for the determination of D-galactose was from 10 μM to 14 mM, and the recovery from spiked serum, samples at low galactose levels, was close to 100%. The sample frequency was 45 h^{-1} and the sample volume was 160 μl . The sampler, the pump and the valves in the flow system were controlled by a personal computer which also collected and analyzed the data.

INTRODUCTION

Determination of D-galactose is of interest in medicine, e.g. for liver tests [1], test for galactosemia [2], and for the study of glucose intolerance in newborns [3]. It is usually determined through a catalytic reaction involving galactose oxidase. The enzyme also acts on a range of substrates with terminal D-galactopyranosyl residues, e.g. raffinose, lactose and other compounds of interest in

the food industry [4,5]. The efficiency of the oxidation of dihydroxyacetone [6] might be utilized for biotechnical process control [7].

Galactose oxidase (GalOD) catalyzes the reaction between D-galactose and oxygen



in a fairly complex reaction the details of which are only partially understood [8,9]. The enzyme contains copper [10], which seems to change between Cu(III) and Cu(I) during the course of Eqn. (1) [11]. An infrequent side reaction produces an inactive form of the enzyme with an intermediate oxidation state which may be Cu(II). The enzyme can be reactivated by a number of oxidants, including hexacyano-ferrate(III). The transition between the Cu(III) and the Cu(II) forms of the enzyme is mediated by certain redox couples like hexacyano-ferrate(III) and hexacyanoferrate(II). An induction period which is observed in phosphate buffers is eliminated in the presence of e.g. hydrogen peroxide and hexacyanoferrate(III).

The enzyme may loose its copper and become an inactive apoenzyme but the activity can be restored by treatment with a copper(II) solution [12]. A number of substances can inhibit the enzyme, the most important in practice are the inhibition by high concentrations of chloride, hydrogen peroxide and hexacyanoferrate(III). Hydrogen peroxide, which is formed during turn-over, may thus, depending on the conditions, act as an activator or an inhibitor.

The enzyme is reported to be stable at room temperature over extended periods of time, especially in phosphate buffers. It has a good natural resistance towards bacterial attacks, elevated temperatures and organic solvents.

Galactose oxidase is the most commonly used enzyme for galactose determinations, although galactose dehydrogenase also seems to work well [13]. The reaction, Eqn. (1), can be followed by monitoring the consumption of oxygen [12] or the production of hydrogen peroxide

which can be detected amperometrically [2,14], fluorometrically [1] or spectrophotometrically [15,16]. Of special interest in connection with this work is a flow injection determination (f.i.a.) using immobilized galactose oxidase and a Clark electrode [12].

EXPERIMENTAL

Preparation of the galactose oxidase reactor

Galactose oxidase (EC 1.1.3.9) was obtained from Sigma Chemical Co. and both type IV and type V, which differ in purity, have been used. Attempts to immobilize the crude enzyme were unsuccessful and a purification was therefore undertaken. The enzyme, 400 Sigma units, was dissolved in 1 ml buffer and dialyzed against phosphate buffer, pH 7.0, overnight and against 20 mM sodium acetate, pH 5.0, for 30 min. The dialyzed solution was injected directly into a preparative HPLC-system containing a 5 mm i.d. Mono-S HR 5/5 cation exchanger column (Pharmacia Fine Chemicals, code no. 17-0547-01) and a spectrophotometric detector. Elution (1 ml min^{-1}) was made with a gradient of 0 - 0.5 M NaCl in the sodium acetate buffer. The gradient (20 mM min^{-1}) was applied after all the unretained material had been eluted. The galactose oxidase fraction (0.8 ml) contained about 90% of the oxidase activity of the injected sample and the specific activity (units/A_{280}) had increased 32 times. The fraction was immediately dialyzed against 0.25 M phosphate buffer, pH 8.5.

The dialyzed galactose oxidase fraction was added to 50 mg arylamino-glass activated as described before [17,18] and allowed to react overnight at room temperature. Almost 100% of the enzyme was retained on the support. The glass (Electronucleonics Inc.) had a pore size of 33 nm and a particle size of 75-125 μm . The enzyme-glass was packed into a Teflon tubing, i.d. 1.9 mm, volume 95 μl , fitted with retaining nets made of polypropylene. The reactor was provided with Altex screw fittings for connection to other components in the flow system. The enzyme reactor was stored in a Tris buffer containing 2 mM CuSO_4 and 0.1 mM EDTA.

Preparation of the other reactors in the f.i.a. system

Peroxidase (EC 1.11.1.7, Sigma type VI, P-8375) was immobilized to diazotized CPG-glucophase-G (Pierce Chemical Co.) with a pore diameter of 46 nm and a particle size of 37-74 μm . The support was diazotized with the same procedure as used earlier for alkylamino-glass [18] and described elsewhere [19]. 2.5 mg enzyme was taken to 50 mg glass and the coupling yield was 2%. The enzyme-glass was packed into a reactor, i.d. 2.4 mm, volume 180 μl . The apparent pseudo first order rate coefficient of the reactor (K_{POD}) was 1.1 s^{-1} and the conversion of hydrogen peroxide to products should therefore be at least 99.99%. The low loading and large reactor volume is advantageous for the colour-forming reaction [20].

The coloured products which form in the peroxidase reactor will adsorb on normal hydrophobic porous glass and the adsorption may result in severe peak broadening [21]. The adsorption to the glucophase-G support is negligible under the conditions used in the f.i.a.-system. Extended experiences with the peroxidase reactor indicate that it can be used for several months without visible deterioration.

Catalase (EC 1.11.1.6, Sigma C-40) was also coupled to a diazotized CPG-glucophase-G support material with the same procedure as described for peroxidase: about 25% of the enzyme was retained on the support. The enzyme-glass was packed into a reactor, i.d. 1.9 mm, volume 70 μl . The apparent pseudo first order coefficient of the reactor was $> 2 \text{ s}^{-1}$ corresponding to $> 99.99\%$ destruction of the hydrogen peroxide.

Bond-Elut-NH₂ (Analytichem International, Harbor City, CA 9071), is a disposable column packing for sample clean-up. The material, which consists of propylamine silylated silica, was packed into a Teflon tube, i.d. 1.9 mm, volume 70 μl , with methanol as a solvent. The column was washed with water, saturated with copper, (10 mM CuSO₄, 15 min), washed with a salt solution (10 mM NaCl, 10 min), and with 0.1 mM ascorbic acid (15 min), at a flow rate of 1 ml min⁻¹. The latter treatment removes loosely bound copper. The capacity for

ascorbic acid removal after this treatment corresponds to 10 μ mole as determined by breakthrough curves. A new reactor was taken each morning and the used reactors were discarded because of some slow irreversible inactivation.

F.i.a. manifold for galactose determination

The sample loop (160 μ l) of a pneumatic injection valve (Cheminert, mod. SVA 8031) was filled by a suction pump from a sampler with test tubes or vials (Fig. 1). The sample was injected into a carrier stream normally consisting of water, and dialyzed on-line in a home made dialyzer [22], channel lengths 475 mm, channel widths 1.5 mm, and channel heights 0.25 mm. The dialyzer consisted of two equal Perspex halves with cut channels and they were clamped together with a 0.025 mm thick cellulose acetate dialysis membrane (Elkey Products Inc., Worcester, Mass., Cat. no. DM-C-2-157-0144) in between. A restrictor was inserted at the waste outlet to match the back pressure on the detector side of the dialyzer.

The channels of the dialyzer could be filled with solid glass beads, 150-250 μ m, to reduce the dispersion. Filling was facilitated by wetting the channels with a water soluble adhesive (Triton-X). The filling was made with the dialyzer in open position and a dry membrane was placed between the halves before they were clamped together. Polypropylene nets had been mounted at the outlets to retain the glass beads.

The dialyzer was followed by the Bond-Elut-NH₂-Cu column for the removal of interferences and by a catalase reactor for removal of hydrogen peroxide from the sample and from reactions in the Bond-Elut-NH₂-Cu reactor (Fig. 1). The hydrogen peroxide, which is produced by oxidation of galactose in the galactose oxidase reactor, was monitored through the color which is formed in an oxidative coupling reaction in the peroxidase reactor. The final detection was made spectrophotometrically at 514 nm (LKB HPLC-detector mod. 2051). A column containing about 200 μ l arylamine activated porous glass was inserted into the reagent line to adsorb impurities. The carrier and the reagent streams were pumped by a 4-channel peristaltic pump

(Gilson, Minipuls 2). The confluence points were followed by short single bead string reactors [23] for efficient mixing. All tubings were of Teflon, i.d. 0.5 mm.

The operation of the f.i.a. system was controlled from a personal computer (Luxor AB, mod. ABC 800, with high resolution graphics) with interfacing cards for the sampling pump and for the pneumatics for the sampler and the injector. The detector output was connected to an AD-converter so that single peaks could be displayed on the whole screen in parallel with the recorder. The peak heights and areas were evaluated and stored for further numerical handling with e.g. statistical methods.

Chemicals

A number of reagents have been in common use for peroxidase-catalyzed detection of hydrogen peroxide. The requirements on the reagent differ in some respects when an immobilized or a soluble enzyme is used. The combination of 2,4-dichlorophenol-6-sulphonate (DCPS), and 4-aminoantipyrine (4-AP) seems to be well suited for f.i.a.-applications [19]. The DCPS was synthesized as described by Barham and Trinder [24] and it contains unreacted dichlorophenol (DCP) which act as mediator in the peroxidase reaction [25]. The reagent stream contained 20 mM DCPS/DCP, 0.8 mM 4-AP and 1 mM EDTA in 0.1 M phosphate buffer, pH 7.0. The EDTA improves the stability of the reagent against trace metal catalyzed air oxidation so that it can be used for up to a week. The reagent was degassed in order to prevent air bubble formation in the cuvette.

The reaction conditions in the galactose oxidase reactor are controlled by mixing the dialyzed sample with a stream of pH 7.0 buffer. Two buffers were used, namely 0.3 M phosphate or 0.5 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) to which redox controlling reagents were added as described in Results and discussion. Many buffer ions, including TRIS and acetate, will inhibit the enzyme, but a zwitterionic buffer made with N,N-bis(hydroxyethyl)glycine (BICINE) has been reported to be favorable since it eliminates the induction period of the galactose oxidase observed

in phosphate buffers [8]. The zwitterionic buffer HEPES was selected by us since it has a more favorable pK_a and a weaker copper chelating strength than BICINE.

RESULTS AND DISCUSSION

Column activity of the galactose oxidase reactor

Inadvertently coimmobilized catalase in the galactose oxidase reactor will decompose some of the hydrogen peroxide formed in Eqn. (1) and decrease the final colour yield. Even small amounts of catalase will have large effects due to its very high turn-over rate compared to that of galactose oxidase.

The kinetics of hydrogen peroxide decomposition is of pseudo first order and the conversion rate can thus be expressed by

$$-\frac{dC}{dt} = K_{\text{catalase}} \cdot C \quad (2)$$

The integral expression for a plug-flow reactor is

$$\frac{C}{C_0} = \exp(-K_{\text{catalase}} \cdot \tau) \quad (3)$$

where C_0 and C are the substrate (H_2O_2) concentrations at the inlet and outlet of the reactor respectively and τ is the mean residence time. K_{catalase} , which is the apparent pseudo first order rate coefficient of the reactor, is dependent on the concentration and the present rate constant(s) of the enzyme and on factors that effect the utilization of the activity like e.g. the pore and particle size of the support [26].

K_{catalase} of the galactose oxidase reactor was determined from Eqn. (3) with a steady-state flow of hydrogen peroxide through the reactor. The remaining fraction of substrate was measured at the outlet. K_{catalase} was independent of the flow rate and thus of the mean residence time, confirming the validity of Eqn. (3).

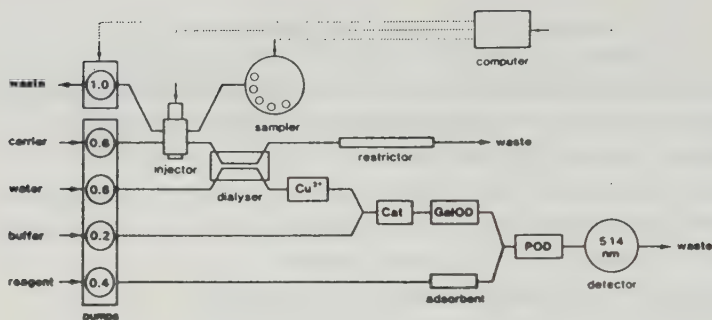


Fig. 1. Schematic diagram showing the f.i.a. system for galactose determination with spectrophotometric detection. The flow rates given in the pump symbols are in ml min^{-1} . Cu^{2+} = Bond-Elut- NH_2 -Cu, Cat = Catalase, GalOO = galactose oxidase and POD = peroxidase reactors. Adsorbent = arylamino porous glass

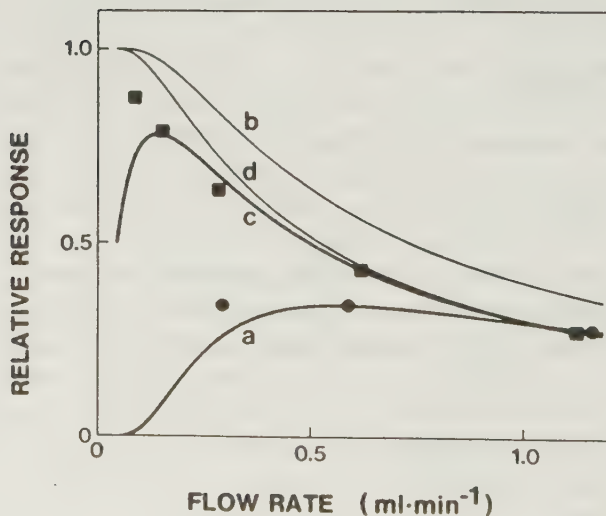


Fig. 2. The responses of a galactose oxidase reactor, a) before treatment; b) hypothetical response of the reactor in a at a catalase activity of zero; c) the same reactor as in a after AT treatment; d) hypothetical response of the reactor in c at a catalase activity of zero.

K_{GalOO} : a,b) 0.028 s^{-1} ; c,d) 0.020 s^{-1}

K_{catalase} : a) 0.034 s^{-1} ; b,d) 0; c) 0.002 s^{-1}

The oxidation of D-galactose in the reactor should be of pseudo first order for substrate concentrations well below K_m ($K_m^{\text{galactose}} = 38 \text{ mM}$ in air saturated solutions [8]) and Eqn. (3) should then be followed also for this reaction. The concentration of hydrogen peroxide at the outlet of the reactor will however depend on the activity of both galactose oxidase and catalase. If the hydrogen peroxide is treated as a metastable intermediate of two consecutive first order reactions, the relative concentration at the outlet ($[H_2O_2]/C_0$) will be [27]

$$\frac{[H_2O_2]}{C_0} = \frac{K_{\text{GalOD}}}{K_{\text{catalase}} - K_{\text{GalOD}}} \left\{ \exp(-K_{\text{GalOD}} \cdot \tau) - \exp(K_{\text{catalase}} \cdot \tau) \right\} \quad (4)$$

where C_0 now is the concentration of D-galactose at the inlet and K_{GalOD} is the pseudo first order rate coefficient for the galactose oxidation.

Since K_{catalase} was determined separately and the mean residence time is known, K_{GalOD} is the only unknown variable. The value of K_{GalOD} was found by fitting Eqn. (4) to the experimental data (Fig. 2). The greatest weight was put on the data at the highest flow-rate where the effect of catalase is minimal.

Fig. 2, curve a, shows the response for a reactor with rather high catalase activity. Curve b represents a simulated case for the same reactor assuming the catalase activity to be zero. It can be seen that it is of importance for the sensitivity of the method to find methods to decrease the catalase activity.

Normal inhibitors for catalase, like cyanide and azide, cannot be used since they will also inhibit the galactose oxidase [5,6,28]. AT (3-amino-1,2,4-triazole) has been used as a ligand in studies of catalase and has been reported to bind irreversibly to the enzyme during turn-over [29]. The reactor used to obtain curve a in Fig. 2 was subjected to an AT treatment (0.1 M AT and 2 mM H_2O_2 , pH 7.0 for 3 h) and the activity of catalase and galactose oxidase was measured again. Curve c in Fig. 2 is the fitted curve for the combined effects of the enzymes while curve d again is the simulated

response of a hypothetical reactor with no catalase activity. It can be seen that the catalase activity of the reactor is reduced to a tolerable level after the AT treatment, but at the expense of a small loss of galactose oxidase activity (compare curves b and d). The net result is nevertheless an improved yield of hydrogen peroxide, compare curves a and c. It was generally found that the curves deviate from the experimental points at low flow rates. This could be due to a weak inhibition of catalase by a product formed in the enzymatic reactions.

AT-inhibited catalase was found to regain its activity slowly and since repeated treatments with AT deactivates the galactose oxidase it should be more profitable to purify the enzyme preparation further.

Medium for the galactose oxidase reaction

The composition of the buffer is known to be important for the activity, for the stability and for the type of kinetics of the enzyme [8]. Two different buffer systems were selected for this study, namely HEPES and phosphate and additions of hexacyano-ferrate(III) and hexacyanoferrate(II) were made to both.

The HEPES buffer was by itself able to activate the enzyme from a low activity state. The enzyme could be brought up to full activity by soaking the reactor in 50 μM hexacyanoferrate(III) in HEPES-buffer. In the presence of D-galactose, i.e. during turn-over, there was, however, a slow deactivation with time. In contrast to this it was found, that the presence of galactose could raise the activity of the enzyme from a low state. A certain activity level was thus associated with each galactose level and it could be reached from the low or the high side depending on the prehistory of the enzyme. Such a behaviour is not acceptable for a component in a system for quantitative measurements.

It is known that the enzyme activity depends on the redox state of the enzyme and that it can be controlled by mediating redox couples [11]. Attempts were therefore made to fix the activity in the high

state by adding 5 μM hexacyanoferrate(III) to the HEPES buffer but it was found that the combination of hexacyanoferrate(III) and HEPES was strongly inhibitory during turn-over. The activity could be restored completely by activation with hexacyanoferrate(III) in the absence of D-galactose.

Phosphate buffer was therefore selected for further studies and it was found that an enzyme which had been brought to full activity by soaking it in 50 μM hexacyanoferrate(III) in phosphate buffer behaved well in the analytical system. A slow deactivation (about $2\% \text{ h}^{-1}$), made it however necessary to reactivate the enzyme periodically.

Hexacyanoferrate(III) (5 μM) was therefore added to the phosphate buffer and it was found that the enzyme could be kept at a state close to full activity. The activity level of the enzyme changed, however, when D-galactose was added and the level depended on the concentration of galactose. This method of keeping the enzyme activity high was therefore abandoned.

The next attempt involved fixation of the redox potential of the solution by additions of both hexacyanoferrate(III) and hexacyanoferrate(II). The activity level was found to change with the redox potential as described before [11] and a mole ratio of 10:1 was finally selected. It corresponds to approximately 90% of full activity and additions of 4 μM hexacyanoferrate(III) and 0.4 μM hexacyanoferrate(II) were sufficient to make the response factor independent of the amount of galactose in the samples.

Optimization of the f.i.a. system components

A good overall performance of a f.i.a. system and in particular one with several reactors and dispersing elements requires an optimization with a certain type of samples in mind. The galactose determination system shown in Fig. 1 was designed for sensitive measurements in complex matrices of biological origin. Both dialysis and means for controlling reducing interferences have therefore been included. The peak broadening caused by each component individually

TABLE 1

Dispersion and pressure drop of the individual components in the f.i.a.-system at the flow-rates shown in Fig. 1. The peak widths were determined with a catechol solution (20 μL samples), see text.

Component	volume (μL)	width _{0.1} ^{a)} (s)	pressure drop (bar)
injector, manifold and detector	160	10	0.4
dialyzer, with glass beads	130 ^{b)}	18 ^{c)}	0.8
Bond-Elut NH_2 -Cu and Catalase reactors	70+70	16 ^{c)}	0.5+0.5
Gal00 reactor	95	12 ^{c)}	0.55
POD reactor	180	12 ^{c)}	0.45
Total system	715	33 ^{c)}	3.2

a) peak width at 10% of the peak height

b) each channel

c) including the injector, manifold and detector

TABLE 2

Effect of injection volume on the peak heights and the peak widths when measured in the complete analytical system with catechol samples. The flow-rates were those shown in Fig. 1.

injection volume (μL)	normalized peak height	width _{0.1} ^{a)} (s)
20	0.16	34
40	0.29	33
80	0.57	34
160	1	38
300	1.43	47

a) peak width at 10% of the peak height

was measured by injecting 20 μl of a tracer (catechol) when each component was alone in the manifold. The peak width is given at 10% of the peak height, see Table 1. The manifold includes the injector, the tubings and the spectrophotometer and its dispersion is included in the peak widths given for the other components.

A dialyzer with fairly long channels was chosen in order to transfer a sizeable fraction of the galactose from the sample to the detector side and this component will therefore contribute significantly to the overall dispersion. The dispersion in open channels can be reduced by introduction of elements which break up the laminar flow [30]. The dialyzer channels were therefore filled with solid glass beads and it can be seen from Fig. 3 that this almost doubles the peak height and decreases the peak width (at 10% of the peak height) to two thirds of the previous value.

The price to be paid is an added pressure drop in the dialyzer. The total pressure drop becomes high due to the large number of components, but it is still within the range of good peristaltic pumps (see Table 1). With the glass beads in the dialyzer channels 11% of the galactose in the sample channel was transferred to the detector side.

The effect of various sample volumes on dispersion and peak height can be seen in Table 2. The sensitivity increases with sample volume without increased dispersion up to 160 μl and this sample volume was therefore selected for the following. Still the sample frequency could be set to 45 samples h^{-1} with baseline separation. The time from injection to the peak maximum was 68 s.

Removal of interferences

Reducing substances like ascorbic acid are well-known interferents in detection methods based on hydrogen peroxide. Ascorbic acid will react with an almost equimolar amount of the intermediates which are formed in the peroxidase reactor. The error is negative, i.e. less coloured compounds are obtained than in the absence of the interferents.

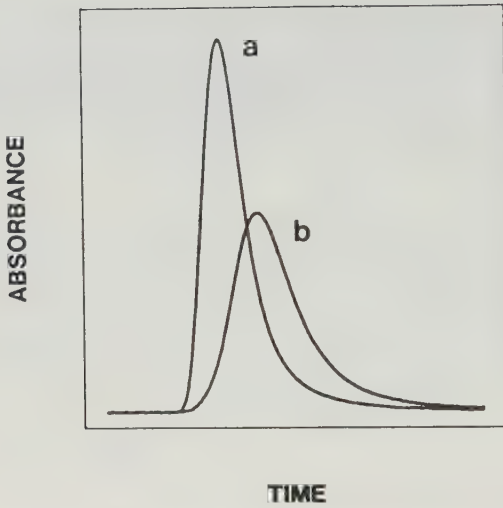


Fig. 3. The peak shape of a dialyzed hydrogen peroxide sample; a) with solid glass beads in the dialyzer channels; b) with open channels.

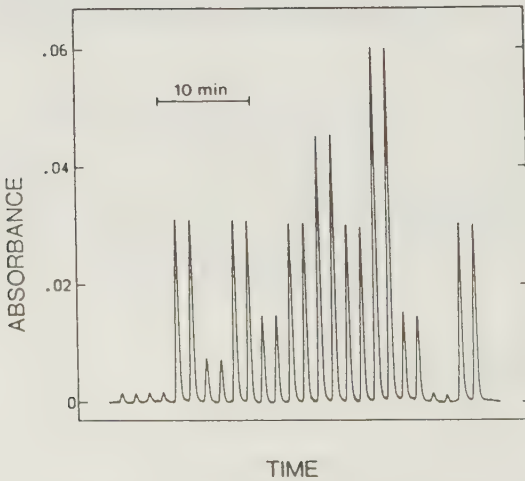


Fig. 4. Response of low-level D-galactose standards. Concentrations from left to right: 10, 200, 50, 200, 100, 200, 300, 200, 400, 100, 10, 200 μ M.

Removal of the interfering substances by alkali deproteinization with a heavy metal salt is an often used method for biological samples [31]. Oxidation by mercury(II) ions has also been described [32]. An off-line step is, however, less attractive than alternative on-line methods like the oxidation by metal-ions bound to an immobilized dithiocarbamate reactor [33].

Effective removal of interferences from added ascorbic acid was achieved by addition of mercury(II) ions (0.5 mM) in the water channel and EDTA (10 mM) in the buffer channel (in a system without the Bond-Elut-NH₂-Cu and the catalase reactors). Because of the waste disposal problem with mercury solutions an immobilized chelate ion exchanger was next tried. The Bond-Elut-NH₂ was found to be equally effective in removing interferences from ascorbic acid with both mercury(II) and copper(II) metal ions. The latter metal was selected for the final system.

Ascorbic acid is oxidized quantitatively to dehydroascorbic acid in the Bond-Elut-NH₂-Cu reactor with the simultaneous formation of hydrogen peroxide. Hydrogen peroxide may also be present in the samples e.g. as a result of aerobic oxidation. The catalase reactor will therefore increase the selectivity independent of the method of reductant removal.

Determination of D-galactose

Some low level galactose standards were run with the described system (Fig. 4). The series with increasing concentration forms a straight calibration curve with a correlation coefficient of 0.9999. Standards with equal, intermediate concentrations (200 μ M), which are interspaced between samples of lower and higher galactose contents, show that no memory effects are present. The results were obtained with a pure phosphate buffer (i.e. no added redox buffer) and they are therefore typical for a system for serum analyses (see below). It can be seen that the peak heights of the 200 μ M series is decreasing slowly due to the formation of the inactive Cu(II) form of the enzyme. The standard deviation calculated without regard to the

successively lowered sensitivity is 1.7% (N=10). The standard deviation of the peaks shown for 10 μ M samples is 5% (N=6).

The measurements were repeated with a system intended for non-serum samples, i.e. with phosphate and hexacyanoferrate(III)/hexacyanoferrate(II) in the buffer line. The sensitivity remained constant with time and the relative standard deviation at the 200 μ M level was typically 0.3-0.5%.

The response to 14 mM galactose is about 2 absorbance units which is close to the upper level of most spectrophotometers. Since the calibration curve is linear up to this concentration the method has a very large linear operating range and it can easily be extended upwards by decreasing the sample volume.

Interferences

The effect of some potential interferents was studied by injection of samples containing the substance with and without added D-galactose (Table 3). The interferences from the tested substances were negligible at concentrations well above those normally expected in serum samples. The reported normal range for e.g. ascorbic acid is thus 0.01-0.1 mM and for total bilirubin 0.003-0.03 mM with up to 0.5 mM bilirubin for newborns.

The effect of sodium chloride injection is due to refractive index changes in the spectrophotometric cell and it disappears with a carrier having the same refractive index as the sample. It is of little concern in practice, however, since the signal due to this effect passes zero and changes sign at the position of the galactose peak maximum. It can be seen that the effect on the response for D-galactose is negligible.

Only a negligible amount of bilirubin is transferred through the dialysis membrane and it is thus effectively kept out of the detection system. In a system without a dialyzer bilirubin is expected to give both chemical and spectral interferences [34].

TABLE 3

Effect of interfering substances on the response when measured separately or when added to a standard solution containing 100 μM D-galactose.

Substance	Concentration (mM)	Relative response ^{a)} (%)	
		alone	added to galactose
none	-	0	100
ascorbic acid	1	0	99
	5	0	100
	10	0	
glutathione	1		100
	5		98
	10	0	
uric acid	1		101
	2	0	
bilirubin	1	0	99
L-cysteine	1		98
methionine	1		96
mixture ^{b)}			100
glycerol	10		200
dihydroxyacetone	0.1	>400	>400
hydrogen peroxide	0.1	0	
sodium chloride	120	5	101

a) The response relative to a 100 μM galactose standard.

b) 1 mM each of ascorbic acid, uric acid, glutathione, L-cysteine and methionine

TABLE 4

Recovery of standard additions of D-galactose to serum.

taken (μM)	Pathonorm B ^{a,c}		Pathonorm B ^{a,d}		pooled serum ^{b,d}	
	found (μM)	recovery (%)	found (μM)	recovery (%)	found (μM)	recovery (%)
0	5	-	9	-	20	-
10			18	90		
50	57	104	57	96	72	104
200	216	106	215	103	226	103
400	406	100	418	102	419	100
600	617	102	640	105	600	97

a) reference serum (Nyegaard & Co. A/S, Oslo)

b) obtained from a local hospital

c) 0.12 M NaCl in the carrier line (see Fig. 1)

d) water in the carrier line

Glycerol and dihydroxyacetone give responses because they are oxidized catalytically by the galactose oxidase. The latter is not expected to be present in serum but normal glycerol concentrations are reported to be up to 0.1 mM. It may thus give a high galactose value if present in abnormal amounts in a sample with low galactose content. Various other substances which are reported to be substrates for galactose oxidase [4,5] are not expected to be present in serum.

Table 4 gives the results obtained when two different sera were spiked with standard additions of D-galactose. One of the sera was measured using two different carriers. The recoveries are close to 100%, although somewhat on the high side. The enhanced response is probably due to the high viscosity of the serum samples compared to that of the aqueous standards used for making the calibration curve.

The galactose levels of the two sera were all very low and close to the limit of the method. The serum galactose levels, which are clinically significant are much higher. A further test was made by removing the galactose oxidase reactor from the system and repeating the measurements of the unspiked sera. No response except for the refractive index effect was observed in the absence of the reactor. The salt effect is absent with the saline carrier and a better low-level response is therefore obtained.

The results given above were obtained with a phosphate buffer. With a buffer containing also hexacyanoferrate(III) and hexacyanoferrate(II) there was a negative response when a serum sample was injected. Several minutes were then required before the normal baseline was reached again. The explanation may be that an unknown dialyzeable component of the serum reacts with one or both of the ions of the redox couple. A buffer containing phosphate alone should therefore be used for serum samples and possibly other clinical samples. The only disadvantage is that more standards have to be included so that corrections can be made for the slowly decreasing galactose oxidase activity.

Phosphate buffers with redox buffering ions give a stable response level and may be preferred in non-clinical application. The background absorbance, which is less than 0.001 with phosphate alone, raises to about 0.015 with the given concentrations of hexacyano-ferrate(II) and hexacyanoferrate(II), which may give a somewhat more noisy baseline for low level measurements.

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v

AMPEROMETRIC DETERMINATION OF GALACTOSE, LACTOSE, AND DIHYDROXYACETONE
USING GALACTOSE OXIDASE IN A FLOW INJECTION SYSTEM WITH IMMOBILIZED
ENZYME REACTORS AND ON-LINE DIALYSIS

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ABSTRACT

A flow injection manifold containing a dialyser and reactors with immobilized galactose oxidase and peroxidase was used for the determination of galactose in urine, lactose in milk and dihydroxyacetone in a biotechnological reaction medium. Hydrogen peroxide is formed in the oxidation of substrates by galactose oxidase. The detection was by amperometric reduction of a mediator which had been produced from the hydrogen peroxide in a reaction catalyzed by the peroxidase. The hydrogen peroxide detection step was studied with several mediators and hexacyanoferrate(II) was selected in combination with a graphite electrode. An ion exchange HPLC procedure was used to purify the galactose oxidase, in particular from catalase, and the kinetics and the selectivity of a reactor containing the immobilized enzyme was investigated. Columns for removal of certain interferents such as ascorbic acid were used for the determination of galactose in urine. The response for galactose standards was linear from the detection limit of 2 μM to 60 mM. The sample throughput was 45 per hour and the relative standard deviation was 0.4%.

INTRODUCTION

The recent progress in the techniques of enzyme immobilization and of flow injection analysis (f.i.a.) can both be utilized in methods for the determination of sugars and some carbohydrates in various types of samples. The enzymes provide the selectivity and the f.i.a. the speed, the automation, and the ruggedness. With a sensitive detector the method can be used over a large concentration interval since the sensitivity can be adjusted by selection of different sample volumes and by changing the dispersion or the dilution.

The flow system concept lends itself to a sectioning of the task into analytical unit operations e.g. dialysis, sample pretreatment, chemical reactions, and final detection. This paper reports on further developments of the components in a flow manifold for the determination of D-galactose, in particular work on a galactose oxidase reactor and on an amperometric detector for hydrogen peroxide.

Galactose oxidase (GalOD) catalyzes the oxidation of D-galactose with the release of hydrogen peroxide [1]



A number of compounds with D-galactopyranosyl residues are also oxidized, e.g. raffinose and lactose, as well as some other polyols, e.g. dihydroxyacetone and glycerol [1-3]. The enzyme is very stable at room temperature in phosphate buffers [4] and it is therefore well suited for immobilization and for use in analytical reactors in flow systems [5,6].

Hydrogen peroxide can be determined amperometrically by direct oxidation [7-9] or by reduction of mediators which previously have been oxidized by the peroxide [10-13]. The reduction of the oxidized form of mediators such as iodide, hexacyanoferrate(II), catechol, and hydroquinone, will take place at weakly reducing electrode potentials.

This approach seems attractive since very low background currents can be obtained and since low applied potentials should decrease the probability of electrochemical interferences.

The reaction between hydrogen peroxide and mediators requires a catalyst and peroxidase is well suited since its reaction with hydrogen peroxide is both very fast and selective. The back-reduction of the oxidized peroxidase is not particularly selective [13] and it is therefore necessary to use a large excess of the chosen mediator in order to promote its oxidation over the oxidation of other components emanating from the samples.

Many real samples require some pretreatment before entering the main enzymatic reactors. On-line dialysis can remove particulate, macromolecular as well as some low molecular weight compounds and dialysers have proved to be very useful in analytical flow systems if appropriately designed [6,14]. Some reducing substances which pass the dialyzer may interfere with the detection and an on-line oxidizing reactor [6,15], has therefore also been added in one of the applications.

EXPERIMENTAL

Amperometric detection

The cell used in this work was of the confined wall-jet type, see Fig. 1. The working electrode was made by pressing rods of graphite (diam 3.0 mm) into a Teflon holder so that only the circular end surface contacted the solution. Spectrographic graphite, type RW 003 from Ringsdorff-Werke GmbH, was cut into pieces and the end surfaces were polished on fine, wet emery paper. Carbon film electrodes were made by vapor deposition from hydrocarbons as described by Lundström [16]. Both electrode materials were pretreated by oxidation at +1.5 V for 30 s in a pH 7.0 buffer.

An Ag/AgCl electrode was inserted into a small chamber in the inlet Teflon block. It was filled with 0.1 M KCl from a reservoir consisting of a syringe. Contact with the sample solution was through

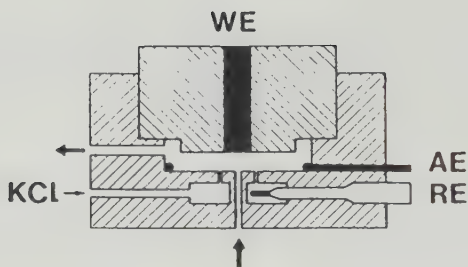


Fig. 1. Diagram of the amperometric flow cell. WE = working electrode, EA = auxiliary electrode, RE = reference electrode. KCl = reservoir of RE solution. For details see text.

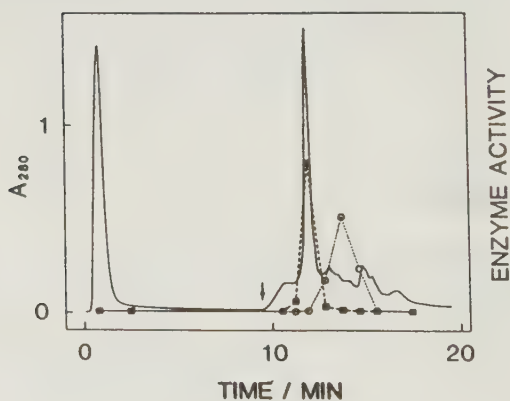


Fig. 2. Separation of galactose oxidase by cation exchange chromatography. — A_{280} , ■—■ galactose oxidase activity, ○··○ catalase activity. The arrow indicates the start of the salt gradient.

four very fine holes located symmetrically around the inlet nozzle. The reference electrode was found to be 39 mV more positive than a saturated calomel electrode (SCE). The auxiliary electrode was a platinum wire wrapped around the circumference of an annulus in the outlet part of the cell. A PAR M-174 potentiostat was used and its output was connected in parallel to a potentiometric recorder and a personal computer.

The response factor varied with the distance between the inlet nozzle and the working electrode but it remained essentially constant from about 0.8 mm to a few mm and a distance of 1.0 mm was therefore selected.

Purification of galactose oxidase

Galactose oxidase (EC 1.1.3.9, Sigma Chemical Co., type V, from *Dactylium dendroides*, 1200 U) was dissolved in 1.2 ml 0.1 M sodium phosphate, pH 7.0 and dialyzed against the same buffer for four hours. It was next dialyzed for 30 min against 20 mM sodium acetate, pH 4.6 and this treatment caused some impurities to precipitate.

The supernatant was injected into an HPLC-system containing a cation exchange column (Mono-S, HR 5/5, Pharmacia Fine Chemicals) and a spectrophotometric detector set at 280 nm. Isocratic elution (1 ml min^{-1}) with a 20 mM sodium acetate, pH 4.6, mobile phase, was used to rinse the column from unretained material. A salt gradient (0–0.5 M sodium chloride in the acetate buffer, gradient slope: $20 \text{ mM sodium chloride min}^{-1}$) was next applied. Fractions were collected and the catalase and galactose oxidase activities of the fractions were determined. The resulting chromatogram with the enzyme activities plotted on the same time scale is shown in Fig. 2. The most prominent peak contained the galactose oxidase and a peak which emerged later contained the catalase. The galactose oxidase fraction (1.6 ml) contained 90% of the oxidase activity of the injected sample and less than 1% of the total catalase activity. The specific activity (U/A^{280}) had increased six times.

Preparation of packed-bed reactors

The above-mentioned galactose oxidase fraction was dialyzed against 0.25 M sodium phosphate, pH 8.5 for 1.5 h and mixed with 50 mg arylamino derivatized controlled-pore glass (CPG-10, Electro-Nucleonics Inc., pore diam. 33 nm, particle size 75-125 μm) prepared and activated as described before [17]. The reaction was allowed to proceed overnight and the supernatant then contained less than 1% of the galactose oxidase activity. The immobilized enzyme was packed into a reactor, i.d. 1.9 mm, volume 135 μl , made of a Teflon tubing fitted with retaining nets of polypropylene and Altex screw fittings. The reactor was activated each day by soaking the immobilized enzyme with a solution of 50 μM hexacyanoferrate(III) for one minute. The reactor was stored in phosphate buffer, pH 7.0, when not in use.

Peroxidase (EC 1.11.1.7, Sigma Chemical Co., type VI, from horse radish) was immobilized to arylamino derivatized controlled-pore glass (CPG-10, Electro-Nucleonics Inc., pore diam. 73 nm, particle size 75-125 μm) as described before [17], and packed into a reactor (i.d. 1.5 mm, volume 50 μl).

Catalase (EC 1.11.1.6, Sigma Chemical Co., C-40, from bovine liver) was immobilized to arylamino derivatized glycophasic G controlled-pore glass (CPG/460, Pierce, pore diam. 46 nm, particle size 37-74 μm) as described before [6]. The enzyme-glass was packed into a reactor (i.d. 1.9 mm, volume 70 μl).

Bond-Elut-NH₂ (Analytichem International), which is a propylamine silylated silica, was packed into a reactor (i.d. 1.9 mm, volume 70 μl), and the amine groups were saturated with copper(II) as described before [6].

Flow-through dialysers

Dialyser A consists of two perspex halves (85 x 65 x 12 mm) with rectangular grooves (widths 1.5 mm, depths 0.25 mm, lengths 475 mm) cut in the surfaces in a folded pattern. The two halves were clamped

together with a cellulose acetate dialysis membrane (Elkey Products Inc., Cat. no DM-C-2-157-0144, thickness 0.025 mm) in between. The liquid volume of each channel was 180 μ l.

Dialyser B was identical to A except that the channels were filled with solid glass beads (diam. 150-250 μ m) to increase the radial mixing and hence to decrease the axial dispersion. The free volume then decreased to 130 μ l on each side.

Dialyser C was made by cutting a straight channel in each of two small perspex pieces. The channels were 4.6 mm long, 1.5 mm wide and 0.5 mm deep. The dialysis membrane was identical to those used in dialyser A and B. A restrictor was inserted at the waste outlet to match the back pressure on the detector side of the dialyzer.

Flow injection manifold

The flow injection manifold is shown in Fig. 3. The sample was introduced with a computer-controlled pneumatic injector (Cheminert SVA 8031) and the sample loop (10-160 μ l) was filled by a peristaltic suction pump. A slide injector (Cheminert CSA) with an injection volume of 1.5 μ l was used in the determination of dihydroxyacetone. The carriers, the buffer and the reagent were pumped with a four-channel peristaltic pump (Gilson, Minipuls 2). The buffer was 0.5 M sodium phosphate, pH 7.0, and the reagent was a deaerated solution containing 5 mM potassium hexacyanoferrate(II) and 0.1 M sodium phosphate, pH 7.0. All connections were made with Altex screw fittings and 0.5 mm i.d. Teflon tubings. Short single bead string reactors [18] (i.d. 0.8 mm, length 50 mm, bead diam. 0.5 mm) were inserted after each confluence point to improve the mixing.

An injected sample is carried into the dialyzer where fractions of the dialyzable components pass through the membrane. The transferred solutes are carried to the galactose oxidase column where substrates of the enzyme are oxidized through the reaction represented by eq(1). The hydrogen peroxide which is formed will next enter the peroxidase reactor where a corresponding amount of oxidized mediator will be

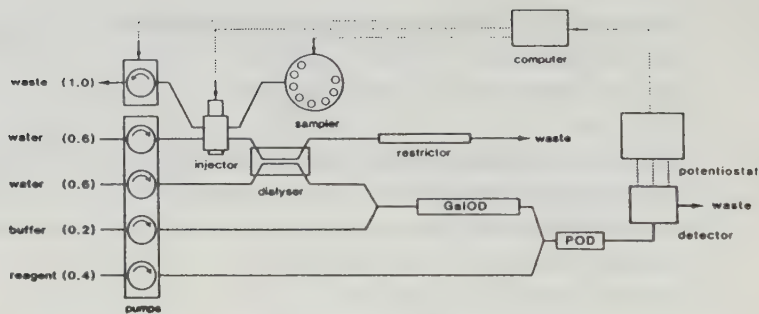


Fig. 3. The f.i.a. manifold. The flow-rates given in the figure are in ml min^{-1} . GalOD; Galactose oxidase reactor, POD; per-oxidase reactor. For details see text.

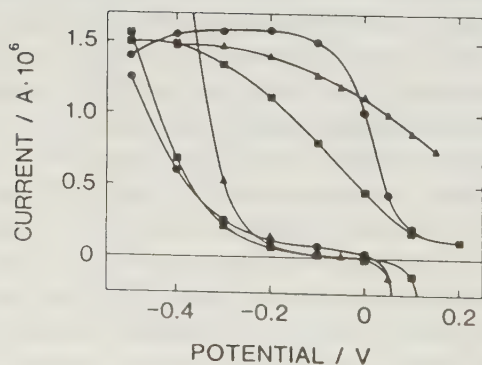


Fig. 4. Current-potential relations in f.i.a. for
 ● oxidized 4-methylcatechol on a graphite electrode
 ▲ hexacyanoferrate(III) on a graphite electrode
 ■ hexacyanoferrate(III) on a carbon film electrode
 lower curves; background currents
 upper curves; baseline corrected peak currents for 80 μl
 injections of 100 μM hydrogen peroxide.

produced. The enzymatically oxidized mediator is then carried to the amperometric cell where it is detected.

Reactors for sample pretreatment were incorporated in the flow system for the determination of galactose in urine. A copper saturated Bond-Elut column was inserted between the dialyser and the buffer confluence point to remove certain reducing compounds [6]. Ascorbic acid will thus be retained on the column and there be oxidized to dehydroascorbate. Hydrogen peroxide is evolved in the process and a catalase reactor was therefore inserted between the buffer confluence point and the galactose oxidase reactor to decompose the peroxide. Copper ions which may be released from the Bond-Elut column interfere with the amperometric detection and 5 mM EDTA was therefore added to the buffer when this column was present.

Chemicals

D-galactose (G 0750), D-raffinose (R 0250), and α -lactose (L 3625) were obtained from Sigma Chemical Co.. Dihydroxyacetone (98%, Merck) was dissolved in water and allowed to monomerize over night. Stock solutions of hydrogen peroxide (Perhydrol, Merck) were standardized by titration with permanganate which in turn had been standardized against oxalate. 4-methyl-catechol (Aldrich) was recrystallized from cyclohexene. All other chemicals were of p.a. quality.

RESULTS AND DISCUSSION

Amperometric determination of hydrogen peroxide

A simplified flow manifold consisting of the injector, the peroxidase reactor and the amperometric cell was used to study the hydrogen peroxide detection step. Hydrogen peroxide was injected and the current-potential relations were determined for two electrode materials and two mediators (Fig. 4).

The reduction of enzymatically oxidized 4-methylcatechol was kinetically well behaved on the graphite electrode as can be seen from the steep rise in current with decreasing potential. The background

currents were rather high which was probably due to the presence of reducible impurities in the fresh 4-methylcatechol solution. This, together with the observation that the solution is unstable and degrades even if deaerated, renders this mediator less suitable.

It can be seen in fig. 4 that the background currents with hexacyanoferrate(II) were very low in a potential range around -50 mV vs the reference electrode at both the graphite and carbon film electrodes. A deaerated solution of hexacyanoferrate(II) is additionally rather stable and the drift in the background current was negligible over a day. The reduction of hexacyanoferrate(III) was quite irreversible on both electrode materials and the limiting current plateau was not reached until rather reductive potentials were applied. The response with the graphite electrode was very reproducible and this combination of electrode material and mediator was therefore selected for most of the measurements reported below.

Some other mediators were also tested. Hydroquinone was found to behave similarly to 4-methylcatechol, whereas resorcinol showed much slower electrode kinetics. A gradual passivation of the electrode surface was also observed and resorcinol was therefore rejected. Iodide was excluded because of adsorption in the peroxidase reactor. A binding of iodine to peroxidase has been reported [19]. The graphite material used in this study was quite reproducible and the pretreatment did not seem to be critical. The carbon film electrode, on the other hand, was quite sensitive to the pretreatment.

The efficiency of the peroxidase reactor was tested by comparing the responses from injections of hexacyanoferrate(III) and hydrogen peroxide. A precise stoichiometry of 1:2 was found which indicates that the conversion of hydrogen peroxide to hexacyanoferrate(III) in the reactor is quantitative. The peroxidase reactor is very stable and it can be used for at least several months without noticeable degradation.

The described flow injection system was operated with the hexacyanoferrate(II) solution and a graphite electrode polarized at -50 mV vs

the reference. The response was linear in the range 10^{-7} - 10^{-3} M hydrogen peroxide with a detection limit of 30-50 nM. The upper limit was set by the limit above which the peroxide was no longer quantitatively reacted with the mediator in the peroxidase reactor.

The immobilized galactose oxidase reactor

The oxidation of substrates by galactose oxidase can be described by Michaelis-Menten kinetics [4]. The kinetic scheme can be simplified to pseudo first order expressions for substrate concentrations well below K_M which for a constant enzyme concentration gives

$$-\frac{dC}{dt} = K \cdot C \quad (2)$$

The integral expression for a plug-flow immobilized enzyme reactor at a steady state feed of substrate is

$$\frac{C}{C_0} = \exp(-K \cdot \tau) \quad (3)$$

where C_0 and C are the substrate concentrations at the inlet and outlet of the reactor respectively, τ is the mean residence time, and K is the apparent pseudo first order rate coefficient for the catalyzed reaction of the reactor, which thus includes the enzyme concentration and the effects of immobilization.

The production of hydrogen peroxide in the reactor by eqn.(1) can be expressed by

$$\frac{[H_2O_2]}{C_0} = 1 - \exp(-K \cdot \tau) \quad (4)$$

where $[H_2O_2]$ is the concentration of hydrogen peroxide at the outlet of the reactor.

Catalase present in the galactose oxidase reactor as an impurity will decompose some of the produced hydrogen peroxide and reduce the net yield of peroxide. The rate of decomposition is directly proportional to the peroxide concentration, and eq(3) should be followed for a

steady-state feed of hydrogen peroxide to the galactose oxidase reactor. When the hydrogen peroxide is produced in situ it can be treated as a metastable intermediate of two consecutive first order reactions, namely the production of peroxide by the oxidase reaction and the destruction of peroxide by the catalase reaction. The solution [20], adapted to a plug flow reactor, is

$$\frac{[H_2O_2]}{C_0} = \frac{K_{GalOD}}{K_{catalase} - K_{GalOD}} \left\{ \exp(-K_{GalOD} \cdot \tau) - \exp(K_{catalase} \cdot \tau) \right\} \quad (5)$$

where K_{GalOD} and $K_{catalase}$ are the apparent pseudo first order rate coefficients of the oxidase and catalase reactions respectively.

Inspection of eq(5) shows that good yields of hydrogen peroxide can be obtained only if $K_{catalase}$ is significantly lower than K_{GalOD} and then only if the residence times are not excessively long.

In a previous study [6] the catalase activity was reduced from an initially high value by periodical treatments with 3-amino-1,2,4-triazole. The treatment also deactivated the galactose oxidase so that the value of K_{GalOD} decreased somewhat after each treatment. The strategy used in this paper was to increase the purity of the galactose oxidase preparation by a more efficient chromatographic procedure before the immobilization. The catalase content of the reactor was thereby reduced to a very low level.

The two rate coefficients in eq(5) can be evaluated from measurements of $[H_2O_2]/C_0$ at different residence times. A simplified flow system, where the dialyser had been removed, was used for the measurements. Large injection volumes were used so that the steady-state response was reached. The flow rate was changed by varying the speed of the pump motor so that the relative flow rates of the channels remained the same. The flow dependence of the amperometric detector was compensated for by comparing the responses at each flow rate with that obtained by hexacyanoferrate(III). It can be seen in fig. 5 that the ratio between detected and injected hydrogen peroxide decreases with decreasing flow rate as the residence time and thus

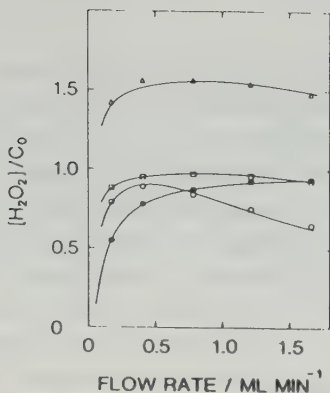


Fig. 5. Yield ($[H_2O_2]/C_0$) of hydrogen peroxide from the galactose oxidase reactor at various flow rates (flow rate through the galactose oxidase reactor) for;

● hydrogen peroxide

○ galactose

□ raffinose

△ dihydroxyacetone

Solid lines are drawn from: ● eq(3) with $K = 0.013 \text{ s}^{-1}$

○ eq(5) with $K_{Gal100} = 0.20 \text{ s}^{-1}$ and $K_{catalase} = 0.006$

s^{-1} , □ eq(5) with $K_{Gal100} = 0.50$ and $K_{catalase} = 0.003$

s^{-1}

The initial concentration of substrates was $100 \mu\text{M}$.

TABLE I

Selectivity of the system with and without dialyser A in the flow manifold.^{a)}

Compound	i_p/C_0 ^{b)} ($\mu\text{A}/\text{mM}$)		$\frac{i_p(\text{with dialyser A})}{i_p(\text{without dialyser A})}$ ^{c)}
	without dialyser	with dialyser	
hydrogen peroxide	2.65	0.250	0.094
galactose	2.33	0.075	0.032
lactose	0.22	0.0041	0.019
raffinose	2.78	0.034	0.012
dihydroxyacetone	4.60	0.278	0.060
glycerol	0.032	0.0018	0.056

a) with $20 \mu\text{l}$ injections

b) peak height divided by the initial substrate concentration

c) lowering of the peak height due to dialyser A

the time for decomposition increases. The recovery of hydrogen peroxide follows eq(3) very precisely and the solid line represents this first order equation with a fitted value of $K_{\text{catalase}} = 0.013 \text{ s}^{-1}$. For galactose and raffinose the yield of hydrogen peroxide increases with decreasing flow rate because the longer residence times allow a larger fraction of the sugars to be oxidized. The yield passes a maximum and then starts to decrease as the decomposition of the formed hydrogen peroxide grows in importance. The solid lines for raffinose and galactose in fig. 5 are drawn from eq(5) with the fitted values of K_{catalase} and K_{GalOD} as given in the figure. The presence of substrate in the reactor apparently decreased the rate of hydrogen peroxide decomposition as indicated by the very low K_{catalase} values obtained in these cases. The experiments were repeated with galactose and raffinose at ten times lower concentrations. The values of K_{GalOD} were unaffected which demonstrates the validity of the first order assumption. The values of K_{catalase} were closer to the value obtained with hydrogen peroxide alone. The precise value of K_{catalase} has however no analytical consequence, since only about 2% of the formed hydrogen peroxide will decompose during the passage through the reactor at the present conditions.

The behaviour of dihydroxyacetone differs from that expected from a simple kinetic and stoichiometric scheme. The high yield of hydrogen peroxide is most likely due to a successive oxidation of the two hydroxy groups. In the simplest case this would lead to a yield ($\text{H}_2\text{O}_2/\text{C}_0$) of almost two, since dihydroxyacetone is a very good substrate for galactose oxidase [3]. Experiments with a smaller galactose oxidase reactor indicate that the rate coefficient for the reaction of dihydroxyacetone is at least as large as that of galactose. This together with the flow dependence suggest that at most 1.6 mole of hydrogen peroxide is formed per mole of dihydroxyacetone under the present conditions. The behaviour of dihydroxyacetone does not reduce the analytical usefulness of the method since the relative yield of hydrogen peroxide is independent of the initial concentration of dihydroxyacetone.

The stability of the galactose oxidase reactor was very good. No noticeable decrease in activity was detected after storage in a refrigerator for one month. The response of the analytical system appears unchanged over a day but a small decrease in the response (0.3% per day) was calculated after the reactor had been continuously used for one month. The stability of the immobilized enzyme was considerably better than the less purified preparation used earlier [6]. No loss of copper from the enzyme was observed and treatments with a copper(II) solution [5,6] had therefore no effect.

Selectivity of the flow injection system and the effect of dialysis

The concentration normalized peak heights, obtained without a dialyser in the f.i.a. manifold, are given in Table I for some compounds. The sensitivity towards a given carbohydrate is affected by the degree of conversion of the compound in the galactose oxidase reactor which in turn is dependent on e.g. the activity of galactose oxidase towards the particular substrate, the activity state of the reactor and the residence time. The relative sensitivities of the various compounds are therefore a conditional measure of the analytical selectivity of the present system. With the dialyser inserted into the flow system the response (Table I) depends on the selectivity of the dialyser as well as on the selectivity of the enzyme reactor. The last row in Table I, which gives the ratio of the sensitivities obtained with and without the dialyser, shows the lowering of the analytical response due to dialyser A, which thus varies for the different compounds.

The peak heights depend on the dispersion caused by the flow components and on the diffusion coefficients of the injected species. Peak area measurements were therefore made with the computer simultaneously with the peak height measurements in order to obtain results which are independent of the peak profile. The net yield of hydrogen peroxide in the galactose oxidase reactor can thus be estimated from the area responses obtained without a dialyser by correlation to the response obtained from injections of hexacyanoferrate(III), see Table II. Very good agreements between the net yields found with the flow

TABLE II

Net yields of hydrogen peroxide and dialysis factors obtained with the f.i.a. system^{a)}

Compound	Q/n_O ^{b)} (As/mmol)	net yield ^{c)} of H_2O_2	Q/n_O ^{b)} (As/mmol)	dialysis ^{d)} factor
	without dialyser		with dialyser A	
hydrogen peroxide	1.62	0.87	0.255	0.157
galactose	1.56	0.80	0.096	0.061
lactose	0.15	0.078	0.0058	0.038
raffinose	1.90	0.98	0.048	0.025
dihydroxy-acetone	3.05	1.57	0.315	0.103
glycerol	0.022	0.011	0.0021	0.096

a) with 20 μ l injections

b) peak area divided by the initial amount of substrate

c) calculated from the peak area obtained from hexacyanoferrate(III) injections

d) in dialyser A

TABLE III

Determination of galactose in urine. For details see text.

Sample	matrix effect ^{a)}	galactose response (mM)	recovery of added galactose (%)
	(mM)		
1 ^{b)}	0.02	0.79	104 ^{c)}
2 ^{b)}	-	0.43	102 ^{c)}
3 ^{b)}	0.02	0.20	97 ^{d)}
4 ^{b)}	0.01	0.24	100 ^{d)}
ascorbic acid (10 mM)	0	0	100 ^{d)}
uric acid (2 mM)	0	0	100 ^{d)}

a) response of the sample with the galactose oxidase reactor removed from the flow manifold (galactose equivalents)

b) urine samples from non fasting individuals

c) 1 mM galactose

d) 0.2 mM galactose

injection system and those found with the steady-state measurements (at the same flow rate) were noticed, compared fig. 5. The number of coulombs per millimole of injected substrate obtained with dialyser A in the flow system is also given in Table II. The ratio between the quantities of electricity obtained with and without the dialyser which corresponds to the dialysis factor, is given in the last row. The high efficiency of the on-line dialyser is demonstrated by the fact that 16% of the hydrogen peroxide and 6.1% of the galactose pass from the sample to the detector channel in a flow system able to process 45 samples per hour. The dialysis factors (as well as the ratio in the last row in Table I) were independent of the concentration of analyte as shown by the linearity of the calibration curves obtained both with and without a dialyser. The size of the dialysis factor decreases with increasing molecular size as expected from theory [21]. The type of compound is also important as shown by the observation that only a very small fraction of hexacyanoferrate(III) passes the membrane.

It was previously found that 11% of the galactose passes the membrane in dialyser B [6] when operated with the same flow rates. This indicates that the more efficient radial mixing caused by the beads in the flow path, apart from reducing the axial dispersion in the dialyser, also enhances the dialysis factor. In certain applications it is, on the other hand, desirable to have a very low transfer of analyte through the dialysis membrane, e.g. when highly concentrated solutions are to be analyzed. An efficient method of reducing the dialysis factor is to shorten the residence time in the dialyser and to decrease the membrane area exposed to the flow paths. With the very small dialyser C only 0.36% of the dihydroxyacetone will pass the membrane at the flow rates stated in fig. 3.

Determination of D-galactose in urine

A flow injection manifold which also contained the Bond-Elut and catalase reactors was used for determinations of D-galactose in urine. With dialyser B and 10 μ l injection of galactose standards the response was linear from the detection limit of 2 μ M up to 60 mM.

This sample volume produces a response corresponding to 7% of the steady-state value obtained with very large injection volumes. The axial dispersion was fairly independent of the sample size up to about 150 μ l. With injections of 160 μ l the detection limit became 0.2 μ M galactose. With samples of this size or smaller 45 samples could be analyzed per hour, with a carry-over of less than half a percent. The relative standard deviation for 80 μ l injections of 100 μ M galactose standards was 0.4% (n=25). The evaluation was made in the computer from baseline corrected peak heights.

Galactose is not present in the urine from fasting healthy individuals. Most of the galactose derived from food intake is converted in the liver but approximately 10% of the galactose is eliminated by renal excretion [22].

The galactose concentration of urine, collected from non fasting individuals, was determined by injection of undiluted samples. The recovery of standard additions of galactose to the samples was also determined (Table III). The good recoveries indicate that the response factor remains the same in the urine matrix as in the aqueous standards. Ascorbic acid, at about the highest concentration reported for urine, and uric acid, at a level set by the solubility, gave no interferences which shows the dynamic efficiency of the Bond-Elut column.

The electrochemical response from unknown sample constituents was measured with the galactose oxidase reactor removed from the flow manifold so that no hydrogen peroxide could be formed from the galactose. A small response was obtained from the urine samples (Table III). Temporary exclusion of the mediator from the reagent indicated that the effect was both due to direct reduction at the electrode and to chemical oxidation of the mediator.

Determination of lactose in milk

The normal concentration of lactose in cow milk is approximately 140 mM and only small amounts of non lactose carbohydrates have been found [23]. Galactose is not present, which makes it possible to use

the present method for lactose determinations, although the response factor (including dialysis) was 16 times lower for lactose than for galactose (see Table I). The determinations were made with dialyzer A in the flow system shown in fig. 3. Milk containing 0.5% and 3.0% fat were analyzed by injecting the undiluted samples. The lactose contents were found from a calibration graph and they were 136 mM and 137 mM respectively. The recovery of lactose added to the milk samples was 98-102% for both types of milk. There was no interference from the matrix and the result was the same for determinations of the lactose concentration in undiluted milk and in milk diluted ten times with water. The linear range of the method with 20 μ l injections was 0.05-300 mM.

Determination of dihydroxyacetone in the presence of glycerol

Dihydroxyacetone is produced enzymatically from glycerol in a biotechnological process with free [24] or immobilized [25] microorganisms. The initial concentration of glycerol is typically 0.5 M and it decreases as the product dihydroxyacetone accumulates. The yield is good and approximately one mole of dihydroxyacetone is obtained from each mole of glycerol.

The flow manifold (fig. 3) was used with dialyser C and the small volume injection valve was used so that 1.5 μ l of the undiluted sample could be injected. A single bead string reactor was inserted after the injector to ensure thorough mixing before the sample entered the dialyser. The resulting dilution of the sample with the carrier is necessary to avoid changes in the dialysis factor due to high but variable viscosity of the samples. The small injection volume, together with the dispersion and dialysis of the sample gave a total dilution of 17 000 times.

The response to dihydroxyacetone alone (fig. 6) was strictly linear in the range 0-0.5 M with a slope of 1.62 μ A/M. A series of solutions with dihydroxyacetone concentrations from 0 to 0.5 M and with glycerol concentrations from 0.5-0 M was prepared so that the total carbohydrate concentration was 0.5 M in each solution. The other constituents of a process reaction medium [26], 50 mM succinate

buffer, pH = 5.0, and 10 mM CaCl_2 were also present in the solutions. The same linear range was obtained (Fig. 6) and the slope became 1.61 $\mu\text{A}/\text{M}$ dihydroxyacetone. The slightly different slope depends on a slow oxidation of glycerol in the galactose oxidase reactor. The response to a solution of 0.5 M glycerol alone corresponds to 4.5 mM dihydroxyacetone and the selectivity of the method against glycerol is thus better than 100:1, compare Table I. Calibration with standards containing both glycerol and dihydroxyacetone can give a correct calibration curve for dihydroxyacetone since the concentration profiles cross in a well defined manner during the course of the biotechnical process. About seventy samples per hour could be processed with negligible carry-over and with a relative standard deviation of less than 0.5%.

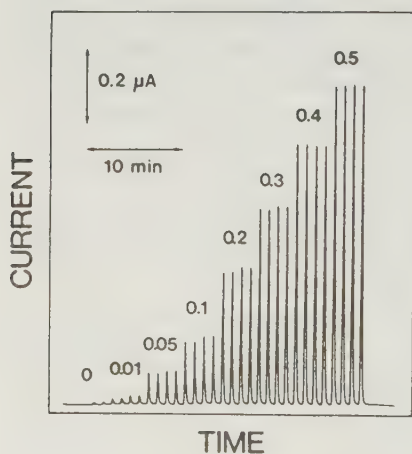


Fig. 6. Flow injection analysis of dihydroxyacetone in the absence and presence of glycerol. The number above the peaks indicates the concentration (M) of dihydroxyacetone. For each concentration a duplicate of dihydroxyacetone alone was injected, followed by a duplicate of dihydroxyacetone with glycerol added to a total carbohydrate concentration of 0.5M.

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